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Metastatic breast cancer cells express high levels of the transforming growth factor β (TGFβ). Although TGFβ is a potent tumor suppressor, it can promote formation of highly metastatic tumors by stimulating an epithelial to mesenchymal transition (EMT), cell migration, and changes in tumor microenvironment. Our initial observation indicated that p38 mitogen activated protein kinase (p38Mapk) is required for these TGFβ responses in epithelial cells. Metastatic breast cancer cells express high levels of active p38Mapk and this contributes to tumor invasiveness. Thus, p38Mapk is a potential target for selective therapeutic intervention. The purpose of this research is (1) to examine the role of p38Mapk in tumor cell motility and invasiveness, and (2) to evaluate the efficacy of p38Mapk inhibitors. This annual report presents data that p38Mapk inhibitors block TGFβ-induced migration of breast cancer cells and fibronectin expression, but do not affect TGFβ growth inhibitory effect. We show that kinase-active TGFβ receptor stimulates p38Mapk activity and cell migration, whereas kinase-inactive receptors inhibit these responses. Thus, TGFβ may contribute to metastasis by increasing cell motility and changes in microenvironement, and p38Mapk inhibitors effectively block these responses.

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TGFbeta, metastasis, p38Mapk, fibronectin, Tak1, cell migration

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Table of Contents

Introduction

Metastatic mammary tumor cells frequently express high levels of the transforming growth factor β (TGFβ). Although TGFβ is a potent tumor suppressor, it can promote formation of highly metastatic tumors by stimulating an epithelial to mesenchymal transition (EMT), cell migration, and changes in tumor microenvironment. The molecular mechanisms of EMT and cell migration induced by TGFβ are not well understood. Our initial observation indicated that inhibition of the p38 mitogen activated protein kinase (p38Mapk) pathway blocked TGFβ-induced EMT and cell migration in NMuMG mouse mammary epithelial cells, suggesting a critical role for this pathway in the tumor promoting activity of TGFβ. Other studies have shown that metastatic breast cancer cell lines express high levels of active p38Mapk and its inhibition reduces invasiveness of tumor cells. Thus, the p38Mapk pathway is a potential target for selective therapeutic intervention. The purpose of this research is (1) to examine *in vitro* the role of the p38Mapk pathway in tumor cell motility and invasiveness, and (2) to evaluate the efficacy of p38Mapk inhibitors on tumor growth and metastasis *in vivo*.

Body

According to the approved Statement of Work we first performed studies stated in Task1.

Task1a: *Examine the effect of p38Mapk inhibitors on spreading, motility, invasiveness and growth of mammary tumor cells.*

We examined the ability of several p38Mapk kinase inhibitors (SB203580, SB202190, PD169316) to block epithelial to mesenchymal transition (EMT) in NMuMG mouse mammary epithelial cells and SiHa human cervical carcinoma cells and to inhibit migration of MDA-MB-231 breast cancer cells and 4T1 mouse mammary epithelial tumor cells. We found that all tested inhibitors blocked EMT in NMuMG and SiHa cells and migration of MDA-MB-231 and 4T1 cells at the concentration 10-15 μM, without inhibition of TGFβ-induced phopshorylation of Smad2 and Smad-mediated transcriptional response (reported in (1)). We also found that p38Mapk inhibitors blocked TGFβ-induced expression of fibronectin, an extracellular matrix protein, which is involved in tumor metastasis (see Figure 1).

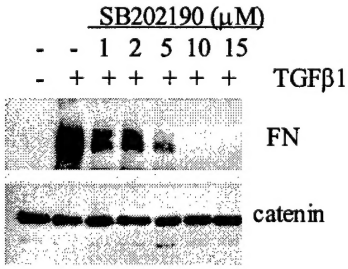

Fig. 1. Fibronectin expression in MDA-MB-231 cells co-treated with 2 ng/ml TGFβ1 and various concentrations of SB202190, a p38Mapk inhibitor.

Task 1b: *Engineer retroviruses expressing Green Fluorescent Protein (GFP) and dominant negative mutants of p38Mapk, Mitogen-activating protein kinase kinase 3 (MKK3), TGFβ-activating kinase 1 (Tak1), TGFβ type I and type II receptors.*

To address this task we sub-cloned TGFβ type I receptor mutants (constitutively active Alk5-T204D, dominant negative Alk5K232R, and wild-type Alk5WT), dominant negative HA-tagged MKK3AL, MKK6AL, Flag-tagged p38AGF in retroviral vector pBMN-IRES-EGFP. The constructs were confirmed by sequencing. Retroviruses were

generated using HEK-293T packaging cells and used to infect MDA-MB-231 and NMuMG cells. GFP-positive cells were selected by FACS. Expression of kinase-inactive TβRII-K277R or Alk5-K232R blocked EMT and activation of the p38Mapk pathway, whereas constitutively active Alk5T204D induced EMT and phosphorylation of MKK3/6 and p38Mapk. Studies with NMuMG cells have been described in (1).

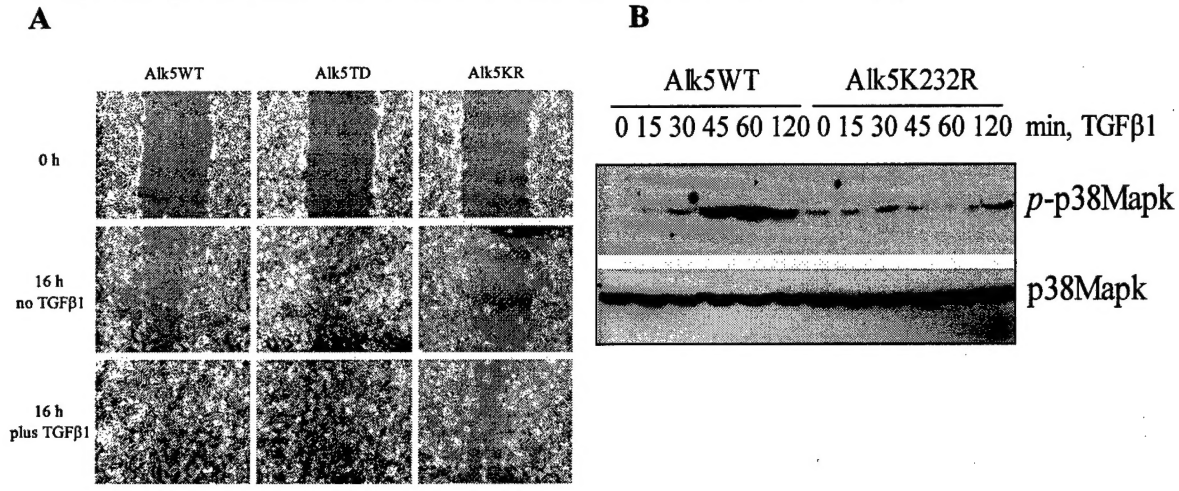

Fig. 2. A. Wound closure in MDA-MB-231 cells expressing wild-type Alk5, kinase-active Alk5T204D, or kinase-inactive Alk5K232R. Where indicated, cells were incubated with 2 ng/ml TGFβ1. **B.** Phosphorylation of p38Mapk in MDA-MB-231 cells expressing Alk5WT or Alk5K232R.

***Task 1c** : Generate cell lines by infection of tumor cells (4T1, EMT6, MDA-MB-231, BT-549) with retroviruses obtained in Task 1b. Characterize cell lines regarding the level of expression of dominant negative proteins and their effect on p38Mapk activity.*
***Task 1d:** Examine the spreading, motility and invasiveness of engineered tumor cells.*

To address **Task 1c and 1d** we examined cell migration p38Mapk activation in MDA-MB-231 cells expressing kinase-mutants of TβRI/Alk5 and TβRII. In MDA-MB-231 cells, kinase-active Alk5T204D enhanced cell migration (Fig.2) and stimulated phosphorylation of p38Mapk (data not shown), whereas kinase-inactive Alk5K232R inhibited these responses (Fig. 2). Similar results were obtained in A431 cells. Kinase-inactive TβRII-K277R blocked phosphorylation of p38Mapk and cell migration (reported in (2)). We also generated MDA-MB-231 cells expressing dominant negative p38AGF, MKK3AL, and MKK6AL. The characterization of these cells is under investigation. In addition, we are currently testing siRNA technology to suppress expression of p38Mapk and MKK3/6 kinases. This novel technology overcomes a drawback of dominant negative approach that requires expression of dominant negative mutants at high levels and may complicate the studies.

Key Research Accomplishments

- We showed that p38Mapk is required for TGFβ-mediated EMT and cell migration.

- We found that p38Mapk is required for TGFβ-induced fibronectin expression in mammary epithelial cells.
- We showed that p38Mapk inhibitors did not block TGFβ-mediated inhibition of cell proliferation.
- We showed that kinase activity of TGFβ receptors is required for TGFβ-induced p38Mapk activation.
- We demonstrated that constitutively active TGFβ type I receptor enhances, whereas kinase-inactive receptor inhibits cell migration. These results show that TGFβ may contribute to tumor metastasis by increasing cell motility.

Reportable Outcomes

Manuscripts:

1. Bakin A., Rinehart, C., Tomlinson A. K., Arteaga C. L., p38 mitogen-activated protein kinase is required for TGFβ-mediated fibroblastic transdifferentiation and cell migration. *J Cell Sci.* 2002 Aug 1;**115**(Pt 15):3193-206.
2. Dumont N., **Bakin** A.V., and Arteaga CL. Autocrine TGFβsignaling mediates smad-independent motility in human cancer cells. *J Biol Chem* 2003 Jan 31;278(5):3275-85.

Abstracts:

1. Bakin, A.V., Rinehart, C. TGFβ regulates expression of target genes and p38Mapk signaling to induce changes in the actin cytoskeleton. AACR, 2003, Abstract #102164.
2. Bakin, A.V., Rinehart, C., Arteaga, C. L. p38Mapk, Sp1 and Smads contribute to TGFβ-induced expression of fibronectin. AACR Special Conference on the role of the TGFbeta superfamily in the pathogenesis of cancer and other diseases, January 15-19, 2003 La Jolla, CA

The following materials have been generated:

1. Retroviral vectors based on pBMN-IRES-EGFP, which encode: TGFβ type I receptor wild type, Alk5-WT, and mutants, Alk5-K232R and Alk5-T204D; dominant-negative (DN) mutants for p38α, p38AGF; MKK3, MKK3AL; MKK6, MKK6AL; Rac1, RacN17; TGFbeta-activated kinase (TAK1), dominant-negative mutant TAK1-K63W; dominant-negative mutant PAK1-N205 (dn-PAK1).
2. Cell lines derived from MDA-MB-231 cells that express wild type and mutants of Alk5 and TGFβ type II receptor; RacN17; p38AGF; MKK3AL; dn-PAK1.

Conclusions

Our studies show that p38Mapk is required for TGFβ-induced migration of breast cancer cells and fibronectin expression, but it is dispensable for TGFβ growth inhibitory effect. TGFβ via receptor stimulates p38Mapk activity and cell migration, whereas kinase-inactive receptors inhibit these responses. Thus, TGFβ may contribute to metastasis by increasing cell motility and changes in microenvironement, and p38Mapk inhibitors effectively block these responses.

References

1. A. V. Bakin, C. Rinehart, A. K. Tomlinson, C. L. Arteaga, *J Cell Sci* **115**, 3193-3206 (2002).
2. N. Dumont, A. V. Bakin, C. L. Arteaga, *J. Biol. Chem.* **278**, 3275-3285 (2003).

Appendices

Reprints of two papers;
Copies of two abstracts;
Curriculum Vitae

p38 mitogen-activated protein kinase is required for TGFβ-mediated fibroblastic transdifferentiation and cell migration

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Summary

Transforming growth factor β (TGFβ) contributes to tumor progression by inducing an epithelial to mesenchymal transdifferentiation (EMT) and cell migration. We found that TGFβ-induced EMT was blocked by inhibiting activation of p38 mitogen-activated protein kinase (MAPK) with H-7, a protein kinase C inhibitor, and with SB202190, a direct inhibitor of p38MAPK. Inhibition of the p38MAPK pathway affected TGFβ-mediated phosphorylation of ATF2, but did not inhibit phosphorylation of Smad2. SB202190 impaired TGFβ-mediated changes in cell shape and reorganization of the actin cytoskeleton. Forced expression of dominant-negative (DN) MAPK kinase 3 (MKK3) inhibited TGFβ-mediated activation of p38MAPK and EMT. Expression of DN-p38α impaired TGFβ-induced EMT. Inhibition of p38MAPK blocked TGFβ-induced migration of non-tumor and tumor mammary epithelial cells. TGFβ induced activation of the p38MAPK pathway within 15 minutes. Expression of TGFβ type II (TβRII) and type I (TβRI/Alk5) kinase-inactive receptors blocked EMT and activation of p38MAPK, whereas expression of constitutively active Alk5-T204D resulted in EMT and phosphorylation of MKK3/6 and p38MAPK. Finally, dominant-negative Rac1N17 blocked TGFβ-induced activation of the p38MAPK pathway and EMT, suggesting that Rac1 mediates activation of the p38MAPK pathway. These studies suggest that the p38MAPK pathway is required for TGFβ-mediated EMT and cell migration.

Key words: p38MAPK, TGFβ, Epithelial-mesenchymal transition, Cell migration, Rac1

Introduction

The transforming growth factor β (TGFβ) family of secreted factors regulates various biological processes, including cell proliferation, differentiation and apoptosis (Massague, 1998). TGFβs signal through cell-surface serine-threonine kinase type II and type I receptors. TGFβ binding to TGFβ type II (TβRII) receptor triggers its associaion with the TGFβ type I (TβRI) receptor (Massague, 1998). TβRII phosphorylates and activates TβRI, which, in turn, phosphorylates receptor-associated (RA) Smads (Smad2 and Smad3). RA-Smads bind Smad4 and translocate to the nucleus where they regulate transcription of target genes (Massague, 1998). In addition to Smads, TGFβ can activate Jun N-terminal kinase (JNK) (Atfi et al., 1997; Engel et al., 1999; Frey and Mulder, 1997), extracellular signal-regulated kinase (ERK) (Hartsough and Mulder, 1995), p38 mitogen-activated protein kinase (p38MAPK) (Hanafusa et al., 1999), and Akt (Bakin et al., 2000).

Smad-dependent signaling has been shown to be required for the antiproliferative activity of TGFβ, and components of this pathway are frequently mutated or silenced in several human cancers (de Caestecker et al., 2000). Tumors, however, frequently express high levels of TGFβ and inhibition of TGFβ signaling has been shown to reduce tumor invasiveness and metastasis (Akhurst and Balmain, 1999; Barrack, 1997; Cui et al., 1996; Hojo et al., 1999). A number of studies provide evidence that TGFβ contributes to tumor cell invasion and metastasis by inducing mesenchymal transdifferentiation in epithelial cells (EMT) and stimulating cell migration (Akhurst and Balmain, 1999; Barrack, 1997; Oft et al., 1998). This TGFβ-mediated fibroblastic transdifferentiation is a complex process associated with alterations in epithelial cell junctions, changes in cell morphology, reorganization of the cell cytoskeleton, expression of fibroblastic markers (fibronectin, vimentin), and enhancement of cell migration (Bakin et al., 2000; Miettinen et al., 1994; Piek et al., 1999b).

The molecular mechanisms of TGFβ-mediated EMT and cell migration are not entirely understood. Studies with TGFβ receptors have shown that a truncated TGFβ/bone morphogenic protein (BMP) type I receptor, Alk2, blocks EMT in mouse NMuMG cells (Miettinen et al., 1994). Adenoviral expression of constitutively active human TβRI/Alk5 together with Smad2/3 can induce EMT in these cells (Piek et al., 1999b). Expression of a dominant-negative truncated form of TβRII decreases the formation of invasive spindle tumours (Portella et al., 1998). Adenoviral expression of Smad2/3 induced EMT only in the context of expression of constitutively active Alk5 (Piek et al., 1999b). Overexpression of Smad7, an inhibitor of Smad-dependent signaling, or dominant-negative Smad3 did not affect the transdifferentiation, arguing against

involvement of Smads in EMT (Bhowmick et al., 2001a). Inhibition of JNK with curcumin (Bakin et al., 2000) or by expression of dominant-negative JNK mutant (Bhowmick et al., 2001a) did not affect EMT. TGFβ did not activate the Ras-Raf-ERK1/2 cascade and MEK inhibitors (PD098059 and U0126) did not block EMT in NMuMG cells (Bakin et al., 2000; Piek et al., 1999b). We have recently shown that the phosphatidylinositide 3-kinase (PI3K)-Akt pathway contributes to EMT at the step of tight junction disruption (Bakin et al., 2000). The role of p38MAPK in TGFβ-mediated EMT has not been studied.

The p38MAPK pathway has been implicated in various biological responses to members of the TGFβ superfamily including TGFβ-stimulated migration of smooth muscle cells (Hedges et al., 1999), neuronal differentiation of PC12 cells induced by bone morphogenic protein 2 (BMP-2) (Iwasaki et al., 1999), growth/differentiation factor-5-induced chondrogenesis of ATDC-5 cells (Nakamura et al., 1999), and BMP-mediated cardiomyocyte differentiation (Monzen et al., 1999). Studies in Drosophila have shown that p38MAPKs are required for wing morphogenesis downstream of decapentaplegic (Dpp), a homologue of TGFβ (Adachi-Yamada et al., 1999). The p38MAPK pathway has also been implicated in TGFβ transcriptional responses (Hanafusa et al., 1999; Kucich et al., 2000; Sano et al., 1999).

The molecular mechanism(s) of TGFβ-induced activation of p38MAPK signaling are not defined. Mammalian p38MAPKs are activated by distinct upstream dual specificity MAPK kinases (MKK), MKK3 and MKK6 (Tibbles and Woodgett, 1999). TGFβ-activated kinase 1 (Tak1) phosphorylates MKK3/6 in TGFβ and BMP signaling (Shibuya et al., 1998; Yamaguchi et al., 1995). In addition, other MKK kinases including p21-activating kinase (PAK1) and mixed-lineage kinase (MLK) have been shown to phosphorylate MAPK kinases (MKK3/6) and induce p38MAPKs (Tibbles et al., 1996; Zhang et al., 1995). p38MAPK downstream targets include MAPK-activated protein kinase-2, mitogen- and stress-activated protein kinase-1 (MSK1), and transcription factors ATF2, CHOP, CREB and MEF2C (Tibbles and Woodgett, 1999). Recent studies have found that p38MAPKs are involved in the control of cell cytoskeleton and cell migration via phosphorylation of paxillin and heat shock protein 27 (HSP27) (Hedges et al., 1999).

In these studies we found that H-7, a protein kinase inhibitor, blocks TGFβ-induced EMT and activation of the p38MAPK pathway in NMuMG mouse mammary epithelial cells. The specific p38MAPK inhibitors, SB203580 and SB202190, impaired TGFβ-mediated changes in cell shape, the actin cytoskeleton, and cell migration. H-7 and the p38MAPK inhibitors blocked phosphorylation of ATF2, but did not inhibit TGFβ-mediated phosphorylation of Smad2. Expression of dominant-negative mutants (DN) of MKK3 or p38α inhibited TGFβ-mediated EMT. We also showed that TGFβ activates the MKK3/6-p38MAPK-ATF2 cascade within 15 minutes and expression of DN-MKK3 blocked TGFβ-mediated activation of p38MAPK and EMT. Kinase-inactive TGFβ type II and type I (Alk5) receptors blocked EMT and the activation of p38MAPK. Forced expression of kinase-active Alk5-T204D induced both EMT and phosphorylation of p38MAPK in NMuMG cells. Alk5-T204D-induced EMT was blocked by a p38MAPK inhibitor. Finally, we demonstrated that forced expression of dominant-negative Rac1N17 blocked TGFβ-induced activation of the p38MAPK-ATF2 cascade and EMT.

Materials and Methods

Antibodies and other reagents

TGFβ1 and tumor necrosis factor α (TNFα) were obtained from R&D Systems. Antibodies to fibronectin, Rac1 and Smad2 were from Transduction Laboratories; antibodies to ZO-1 were from Chemicon; the monoclonal antibody to p38MAPK and rabbit polyclonal to haemaglutinin (HA) epitope were from Santa Cruz Biotechnology, Phalloidin-FITC, phalloidin-Texas Red, and Hoechst 3342 were from Molecular Probes. The β-tubulin-Cy3 antibody was from Sigma. Antibodies to phospho-Ser473 Akt, total Akt, phospho-MKK3/6, phospho-p38MAPK, and phospho-ATF2 were from New England BioLabs, and to C-terminal phospho-Smad2 from Upstate Biotechnology. LY294002, H-7, SB203580, and SB202190 were from Calbiochem. GDP and GTPγS were purchased from Sigma. The GST-ATF2 fusion protein was from New England Biolabs. TBS buffer contained 20 mM Tris-HCl, pH 7.6, 150 mM NaCl. TBST was TBS supplemented with 0.1% Tween 20 (*v/v*).

Cell culture

NMuMG mouse mammary epithelial cells, SiHa human cervical carcinoma cells, MDA-MB-231 human breast cancer cells and HEK293T human kidney cells were purchased from American Tissue Culture Collection (ATCC). Cells were cultured as recommended by ATCC. 4T1 tumor cells were provided by F. Miller (Karmanos Cancer Center, Detroit, MI) and cultured in 10% FBS-DMEM.

Plasmids and retroviral constructs

The retroviral vectors pGabe and pGabe-TβRII-K277R were provided by Martin Oft (UCSF, San Francisco, CA) and have been described previously (Oft et al., 1998). The TβRII-K277R construct contains an HA-tag at the N-terminus. Human wild-type Alk5, dominant-negative Alk5-K232R, and constitutively active Alk5-T204D constructs were provided by Masahiro Kawabata (The Cancer Institute, Tokyo, Japan). To generate pBMN-Alk5 constructs, the *Eco*RI/*Sal*I fragments of Alk5 and Alk5-K232R including the C-terminal HA-tag were cloned in the retroviral pBMN-IRES-EGFP vector provided by Garry Nolan (Stanford University). The pBMN-Rac1N17 was engineered by cloning a *Bam*HI-*Xho*I fragment encoding Rac1N17 from pCDNA3-Rac1N17 (a gift of Richard Cerione, Cornell University, Ithaca, NY) at the *Bam*HI-*Sal*I site of the retroviral pBMN-IRES-GFP vector. RhoAN19 and RhoAQ63L were previously described (Bakin et al., 2000). The pBMN-MKK3AL and pBMN-MKK6AL plasmids were generated by cloning *Sal*I-*Not*I fragment of MKK6AL or *Xho*I-*Not*I fragment of MKK3AL from pCDNA3 vector into the retroviral pBMN-IRES-GFP vector. pCDNA3-MKK3AL and pCDNA3-MKK6AL plasmids were a gift of James Woodgett (The Ontario Cancer Institute, Toronto, Ontario). pBMN-p38AGF encoding a dominant-negative mutant of p38α and containing N-terminal Flag epithope was generated by cloning a *Hind*III-*Xba*I fragment of p38AGF from pcDNA3-p38AGF at the *Xho*I site of pBMN-IRES-GFP. pcDNA3-p38AGF was a gift of Roger Davies (University of Massachusetts, Worcester, MA). Plasmids phCMV-VSVG, encoding vesicular stomatitis virus glycoprotein (VSV-G), and pCMVgag-pol, containing the Moloney murine leukemia virus (MoMLV) gag and pol genes, were provided by Jane Burns (University of California at San Diego).

Retroviral infection of cells

Retroviruses were prepared by transfection of HEK293T cells with 15

μg DNA/100 mm dish of three plasmids encoding gag/pol, VSV-G, and the target construct, ratio 4:3:8. Supernatants from cells were collected for 3 days and combined, filtered through 0.4 μm filters, and stored in aliquots at –80°C. NMuMG cells were infected with supernatant containing retroviruses in the presence of 6 μg/ml Polybrene (Sigma) as described previously (Yee et al., 1994). Three days later, GFP-positive cells were selected by flow cytometry. Under these conditions more than 95% of selected cells expressed GFP at the time of experiments.

Immunoblot analysis

Cells were incubated in serum-free medium for 4 hours prior to treatment with TGFβ1. Cells were lysed in buffer containing 20 mM Tris, pH 7.4, 137 mM NaCl, 1% NP-40, 10% glycerol, 20 mM NaF, 1 mM Na orthovanadate, 1 mM PMSF, 2 μg/ml aprotinin, and 2 μg/ml leupeptin. Protein concentrations in cell lysates were determined by the Bradford method. Protein extracts (50 μg/lane) were separated by 12.5% SDS-PAGE and transferred to nitrocellulose membranes (100 mA, 2.5 hours). Membranes were blocked with 5% milk in TBST for 1 hour at room temperature (RT) and then incubated with primary antibodies in TBST plus 1% milk for 16 hours at 4°C, followed by incubation with secondary antibodies for 1 hour at RT. Membranes were washed three times in TBST and immunoreactive bands visualized by ECL (Pierce).

p38MAPK in vitro kinase assay

p38MAPK was precipitated from protein extracts (200 μg) with a p38MAPK monoclonal antibody (Santa Cruz Biotechnology) for 2 hours at 4°C. An in vitro kinase reaction was performed in a 40-μl volume by adding to the immune complexes 1 μg GST-ATF2 and 10 μCi [γ-^{32}P]ATP (specific activity 3000 Ci/mmol, New England Nuclear) for 20 minutes at 30°C in the presence of 10 μM PKA peptide inhibitor (Calbiochem). Reactions were terminated by the addition of Laemmli loading buffer and heating, followed by 15% SDS-PAGE and transfer to nitrocellulose (NC) membranes. Quantitative analysis of [γ-^{32}P]-labeled bands was performed using a

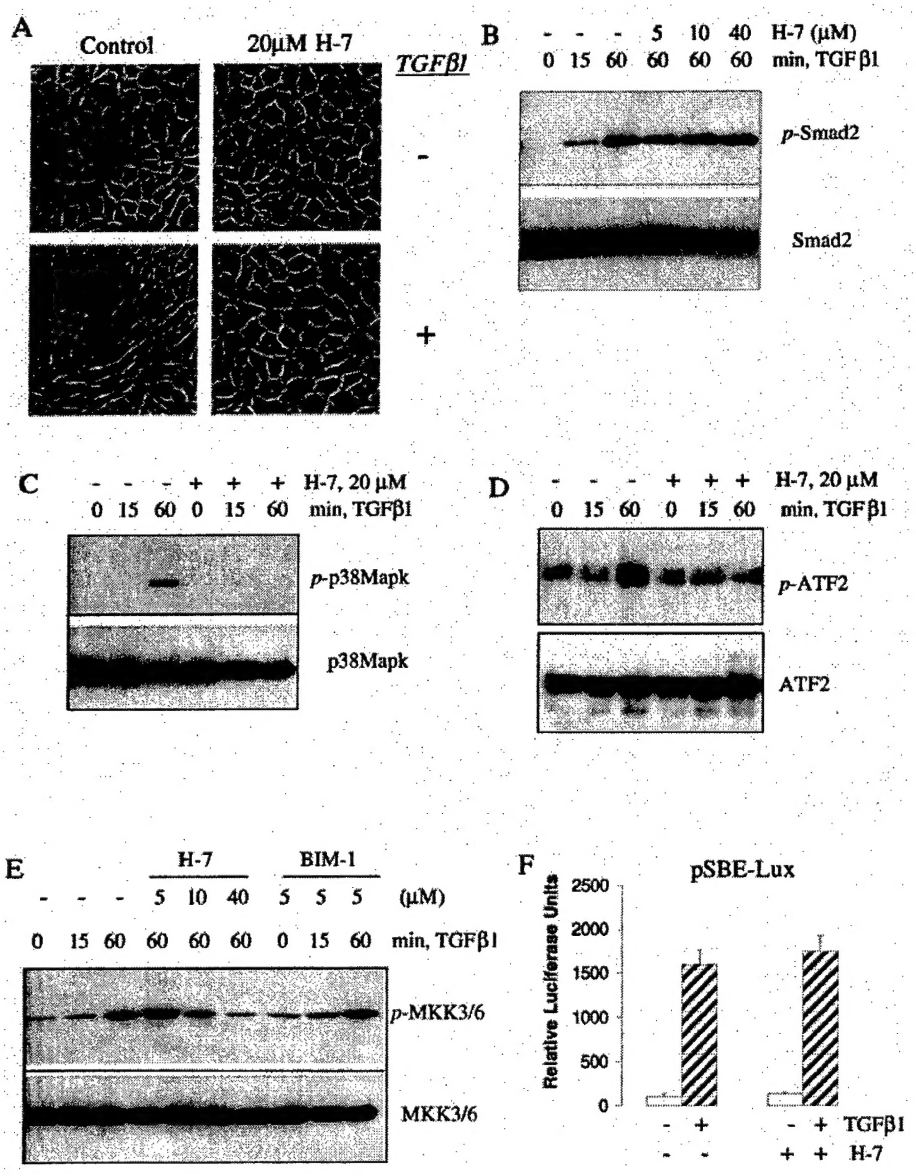

Fig. 1. Inhibition of TGFβ-mediated EMT and p38MAPK activation by H-7 kinase inhibitor. (A) NMuMG mammary epithelial cells were grown on glass coverslips for 24 hours and treated (bottom row) or not (top row) with 2 ng/ml TGFβ1 for 24 hours. Where indicated, cells were co-incubated with 20 μM H-7. Phase contrast images were taken at 200× magnification. (B-E) Immunoblot analysis of whole-cell extracts from NMuMG cells treated with 2 ng/ml TGFβ1 for the indicated times. Kinase inhibitors were added 60 minutes before TGFβ treatment. (B) Immunoblot detection of phospho-Smad2 and total Smad2. (C) Detection of phospho-p38MAPK total p38MAPK. (D) Inhibition of TGFβ-induced ATF2 phosphorylation by H-7. Immunoblots with antisera to phospho-ATF2 and total ATF2. (E) TGFβ-induced phosphorylation of MKK3/6 in cells co-treated with various concentrations of H-7 or 5 μM BIM-I, a PKC inhibitor. (F) Luciferase activity in NMuMG transfected with Smad-dependent reporter pSBE-Lux and pCMV-RI vectors and treated with 1 ng/ml TGFβ1 for 16 hours in the absence or presence of 20 μM H-7. Each bar represents the mean±s.d. of three wells.

PhosphorImager (Molecular Dynamics). The same NC-membranes were probed with a monoclonal antibody to p38MAPK.

Immunofluorescence microscopy

NMuMG cells (10^5cells/well) were grown in DMEM containing 5% FBS on glass coverslips (22×22 mm) for 24 hours before treatment with 2 ng/ml TGFβ1. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes at RT and then permeabilized with 0.05% Triton X-100 for 10 minutes. Cells were washed three times in PBS after each treatment. Cells were blocked with 3% milk in PBS for 30 minutes at RT, incubated for 60 minutes with primary antibodies diluted in 1% milk/PBS (1/300 for ZO-1, 1/500 for Smad2, 1/250 for fibronectin), and then with fluorescent secondary antibodies (1/500) for 45 minutes at RT. Microtubules were stained for 30 minutes at RT with β-tubulin-Cy3 diluted 1/250 in 1% milk/PBS. Actin was stained with phalloidin-FITC (4 units/ml) or phalloidin-Texas Red (2 units/ml). Cell nuclei were stained with 1 μg/ml Hoechst for 10 minutes at RT. Coverslips were mounted on 25×75 mm microslides (VWR Scientific) using AquaPolyMount (Polysciences). Fluorescent images were captured using a Princeton Instruments cooled CCD digital camera from a Zeiss Axiophot upright microscope.

Transcriptional assays

NMuMG cells (3×10^4) were seeded in 24-well plates and transfected with 0.16 μg/ml pSBE-Lux containing 12 repeats of Smad binding sequence (provided by J.-M. Gauthier, Laboratoire Glaxo Wellcome, Les Ulis Cedex, France) with 0.002 μg/ml pCMV-Rl (Promega, Madison, WI) using FuGENE6 reagent (Roche Molecular Biochemicals) according to the manufacturer's protocol. Cells were incubated for 8 hours in 0.5% FBS-DMEM prior to treatment with 1 ng/ml TGFβ1 for 16 hours. Firefly luciferase (Luc) and *Renilla reniformis* luciferase (RlLuc) activities in cell lysates were determined using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). Luc activity was normalized to RlLuc activity and presented as Relative Luciferase Units. All assays were done in triplicate wells and each experiment was repeated at least twice.

Affinity precipitation of Rac using GST-PBD

A fusion protein containing the GTPase-binding domain from human PAK1 (PBD) and glutathione S-transferase (GST) was expressed in *Escherichia coli* using pGEX-4T3-GST-PBD as described (Benard et al., 1999). pGEX-4T3-GST-PBD was kindly provided by Gary Bokoch (Scripps Research Institute). NMuMG cells (2×10^7/assay) were treated with 2 ng/ml TGFβ1 for 15 minutes followed by cell lysis in 20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1% NP-40, 5% glycerol, 20 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 2 μg/ml aprotinin, and 2 μg/ml leupeptin in the presence of 8 μg GT-PBD. Cell lysates were clarified by low speed centrifugation at 4°C. HEK293T cells transfected with Rac1N17 or Alk5 mutants were lysed in the same buffer. After clarification, cell lysates (350 μg/assay) were incubated with 8 μg GST-PBD. To prepare cytosolic Rac1 loaded with GDP or GTPγS, cell lysates (equivalent of 2×10^6 cells) were incubated for 15 minutes at 30°C in the presence of 10 mM EDTA and 100 mM GTPγS or 1 mM GDP to facilitate nucleotide exchange (Benard et al., 1999). The loading reaction was terminated by addition of 60 mM $MgCl_2$. Affinity precipitation was performed using 15 μl of glutathione-Sepharose 4B beads (Pharmacia) for 1 hour at 4°C. The bead pellets were washed three times with 20 mM Tris, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1% NP-40 and 2 times in PBS. The bead pellet was finally suspended in 40 μl of Laemmli sample buffer. Proteins were separated on 15% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with an antibody to Rac1 (Transduction Laboratories).

Migration assays

NMuMG or MDA-MB-231 cells (1×10^5/well) were plated in DMEM/0.5%FBS in the upper chamber of 5 μm pore (24-well) transwells (Costar, High Wycombe, UK) and incubated alone or with 2 ng/ml TGFβ1 in the absence or presence of SB202190. After 16

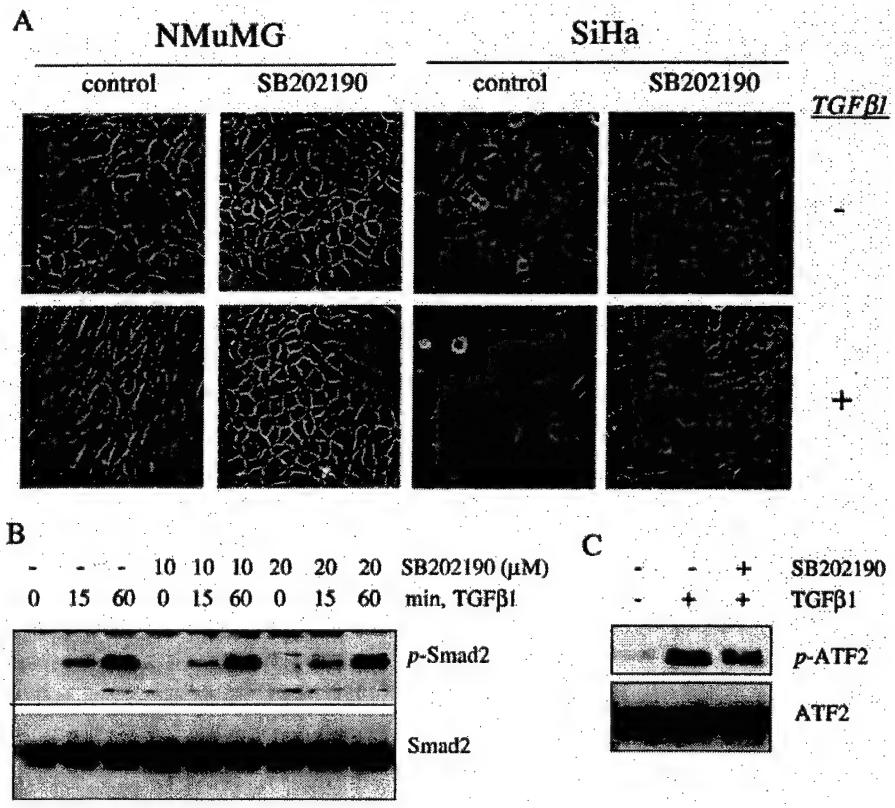

Fig. 2. Blockade of TGFβ-induced EMT by SB202190. (A) NMuMG cells grown on glass coverslips were treated (bottom row) or not (top row) with 2 ng/ml TGFβ1 for 24 hours in the absence or presence of 10 μM SB202190. Phase contrast images were taken at 200× magnification. (B) phospho-Smad2 and total Smad2 immunoblot analysis of whole-cell extracts from cells treated with 2 ng/ml TGFβ1 in the absence or presence of SB202190. (C) Immunoblots with antisera to phospho-ATF2 and total ATF2. SB202190 inhibits TGFβ-induced phosphorylation of ATF2.

hours, cells were fixed in 100% methanol and cells remaining at the top of the polycarbonate membrane were removed with cotton swabs. The cells that had migrated through pores to the lower surface were stained with Diff-quick stain (VWR Scientific). Membranes were mounted on 25×75 mm microslides. Four random images were recorded at 200× magnification and cells were counted. Experiments were performed in duplicate.

Wound closure assay

MDA-MB-231 and 4T1 cells (1-2×10^5/well) were seeded in 12-well plates. Cells were incubated in serum-free medium for 32 hours prior to wounding. The wounds were made by scraping with plastic tip across the cell monolayer. Cells were treated with kinase inhibitors 60 minutes before wounding. The wounded cells were treated or untreated with 2 ng/ml TGFβ1. Phase contrast images were recorded at the time of wounding (0 hours) and 16 hours thereafter. The wound closure was estimated as the ratio of the remaining wound area relative to the initial wounded area. Experiments were repeated at least three times.

Results

H-7 inhibits TGFβ-mediated activation of p38MAPK and EMT

We investigated TGFβ-mediated EMT in NMuMG mouse mammary epithelial cells. These mammary epithelial cells have cuboidal cell shape and form tight and adherens junctions. Treatment with 2 ng/ml TGFβ for 24 hours induced changes in the cell morphology from cuboidal to an elongated spindle-like shape (Fig. 1A). Consistent with previous studies (Miettinen et al., 1994), TGFβ-mediated EMT was blocked in the presence of 20 μM H-7 (Fig. 1A). The inhibitors were added 60 minutes prior to addition of TGFβ and were present during a complete duration of the experiment. Although H-7 has been introduced as a protein kinase C (PKC) inhibitor, it can inhibit other kinases including PKA and PKG (Quick et al., 1992). Therefore, we investigated the effect of H-7 on the signaling pathways induced by TGFβ. We found that TGFβ1-induced phosphorylation of Smad2 was not affected by the presence of H-7 at the concentration that blocks EMT (Fig. 1C). Inhibition of JNK and ERK1/2 did not affect EMT (Bakin et al., 2000; Bhowmick et al., 2001a). Therefore, we tested whether H-7 affects TGFβ-mediated activation of the p38MAPK pathway using polyclonal antibodies to phosphorylated (active) MKK3/6 and p38MAPK. TGFβ-mediated phosphorylation of p38MAPK was blocked in the presence of 20 μM H-7 (Fig. 1C). H-7 also inhibited TGFβ-induced phosphorylation of ATF2, a substrate of p38MAPK (Fig. 1D).

Next, we checked whether H-7 inhibits activation of MKK3/6. We found that TGFβ-induced phosphorylation of MKK3/6 was inhibited by H-7 in a dose-dependent manner (Fig. 1E), suggesting that H-7 inhibits a kinase upstream of MKK3/6. This kinase is downstream of TGFβ receptors as incubation with 5-40 μM H-7 did not block phosphorylation of Smad2 (Fig. 1B). Consistent with this result, H-7 did not block TGFβ-mediated activity of Smad-dependent luciferase reporter (Fig. 1F). Since H-7 can inhibit PKC, we examined activation of p38MAPK in the presence of another PKC

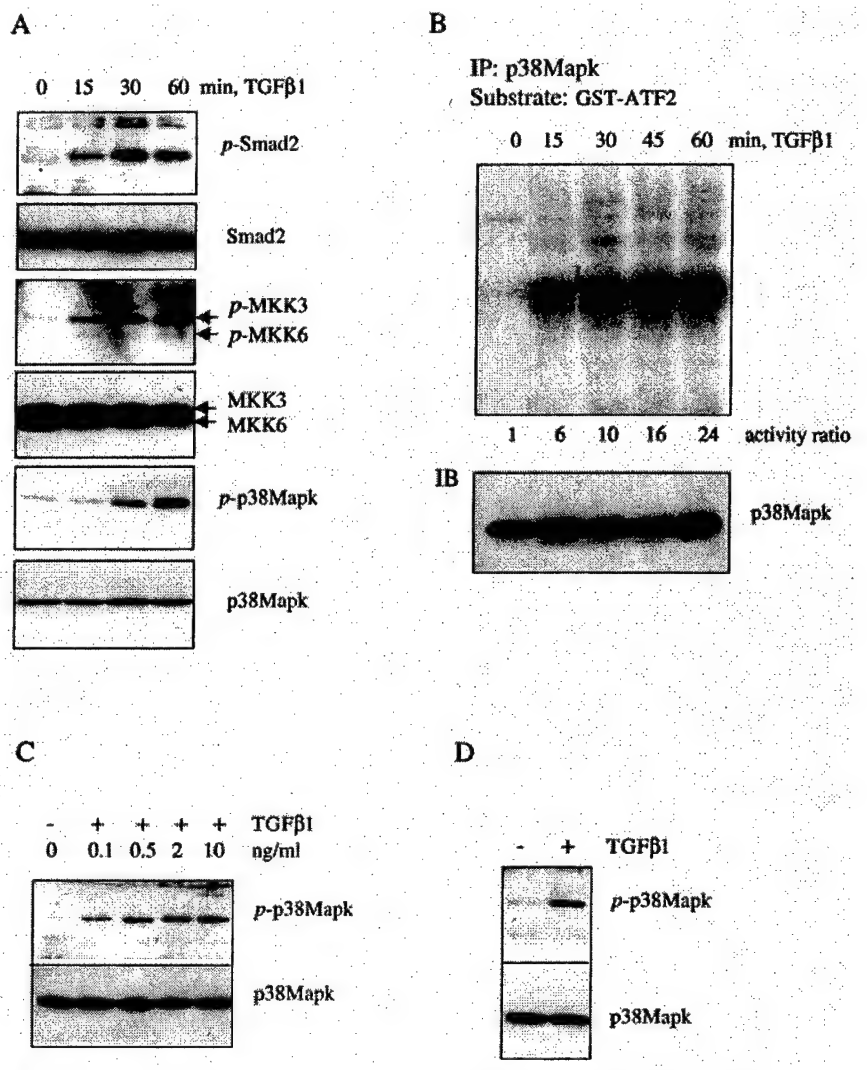

Fig. 3. Activation of the p38MAPK pathway in response to TGFβ. NMuMG cells were incubated in serum-free medium for 4 hours before addition of TGFβ1. (A) Immunoblot analyses with antibodies to phospho-Smad2, phospho-MKK3/6 and phospho-p38MAPK, and total Smad2, MKK3/6 and p38MAPK. (B) Detection of p38MAPK kinase activity in whole-cell extracts from NMuMG cells treated with 2 ng/ml TGFβ1 using GST-ATF2 as substrate. The products were separated by SDS-PAGE and transferred onto nitrocellulose-membrane. γ-^{32}P incorporation into ATF2 was quantitated using PhosphorImager. The membrane was probed with antibody to p38MAPK. (C) Immunoblot detection of TGFβ1 dose-dependent effect on p38MAPK phosphorylation at 60 minutes in NMuMG cells. (D) Induction of p38MAPK phosphorylation by 2 ng/ml TGFβ1 at 60 minutes in SiHa cells.

inhibitor, bisindolylmaleimide-I (BIM-I) (Davies et al., 2000). Treatment of cells with doses of BIM-I (1-5 μM) that block typical PKCs (Davies et al., 2000) did not affect phosphorylation of MKK3/6 in response to TGFβ (Fig. 1D). These results suggest that H-7 impairs TGFβ signaling by inhibiting activation of the p38MAPK pathway downstream of TGFβ receptors, and not through its effect on PKCs.

p38MAPK is involved in TGFβ-mediated EMT

To test whether p38MAPK is involved in EMT, we used specific inhibitors of p38MAPK, SB202190 and SB203580 that do not affect JNK, MEK1/2 and ERK1/2 (Davies et al., 2000). Microscopic examination showed that cell elongation induced by TGFβ in NMuMG cells was blocked by co-treatment with 10 μM SB202190 (Fig. 2A). Similarly, the p38MAPK inhibitor blocked TGFβ-induced cell elongation in cervical cancer epithelial SiHa cells (Fig. 2A). Previous studies have shown that these p38MAPK inhibitors may affect the kinase activity of TGFβ receptors (Eyers et al., 1998). Therefore, we examined their effect on TGFβ-receptor-dependent phosphorylation of Smad2. Treatment of cells with TGFβ in the presence of SB202190 did not significantly affect the expression and TGFβ-induced phosphorylation of Smad2 (Fig. 2B), whereas it

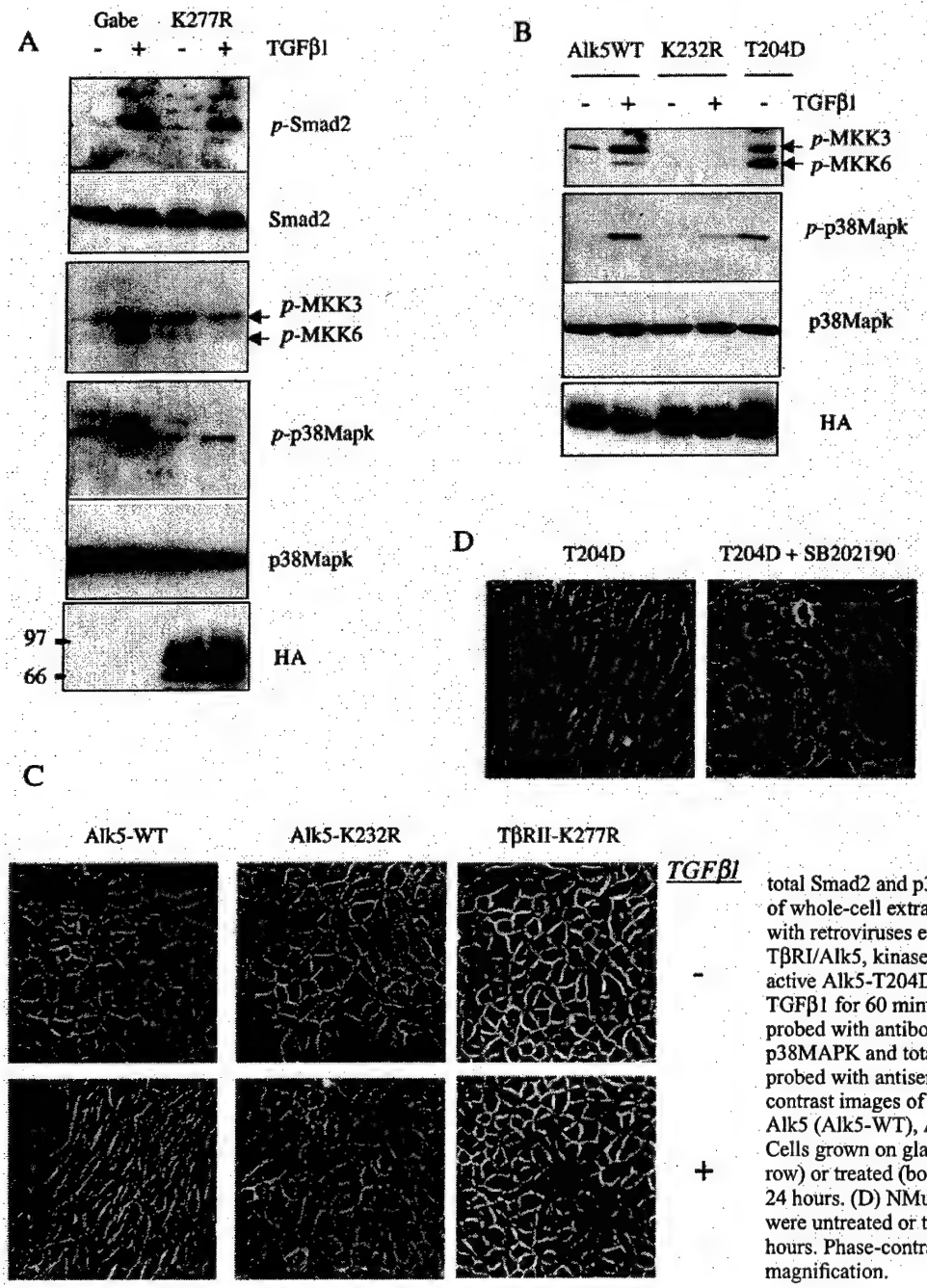

Fig. 4. Effect of kinase mutant TGFβ receptors on TGFβ-induced EMT and activation of the p38MAPK pathway. (A) Immunoblot analyses of whole-cell extracts from NMuMG cells infected with retrovirus encoding TβRII-K277R or control virus (Gabe). Cells were treated with 2 ng/ml TGFβ1 for 60 minutes. Expression of HA-tagged TβRII-K277R was detected with antisera to the HA-epitope. Dominant-negative TβRII-K277R inhibits phosphorylation of Smad2, MKK3/6 and p38MAPK in response to TGFβ. Membranes were re-probed with antibodies to total Smad2 and p38MAPK. (B) Immunoblot analyses of whole-cell extracts from NMuMG cells infected with retroviruses encoding HA-tagged wild-type (WT) TβRI/Alk5, kinase-inactive Alk5-K232R, and kinase-active Alk5-T204D. Cells were treated with 2 ng/ml TGFβ1 for 60 minutes, and protein extracts were probed with antibodies to phospho-MKK3/6, phospho-p38MAPK and total p38MAPK. Membranes were re-probed with antisera to the HA-epitope. (C) Phase contrast images of NMuMG cells expressing wild-type Alk5 (Alk5-WT), Alk5-K232R, and TβRII-K277R. Cells grown on glass coverslips were untreated (top row) or treated (bottom row) with 2 ng/ml TGFβ1 for 24 hours. (D) NMuMG cells expressing Alk5-T204D were untreated or treated with 15 μM SB202190 for 24 hours. Phase-contrast images were recorded at 200× magnification.

reduced phosphorylation of ATF2 (Fig. 2C). Similar results were obtained with SB203580 (data not shown).

TGFβ activates the p38MAPK pathway in NMuMG and SiHa cells

We next examined activation of the p38MAPK pathway in response to TGFβ. Protein extracts were prepared from cells starved in serum-free medium for 4 hours and treated with TGFβ1. Phosphorylation of MKK3/6 was detected after 15 minutes of TGFβ treatment reaching a maximum at 60 minutes, whereas an increase in p38MAPK phosphorylation at Thr180/Tyr182 was observed at 30 minutes and reached a plateau at 60 minutes (Fig. 3A). To confirm the immunoblot data, we tested p38MAPK-specific activity using an in vitro kinase assay with GST-ATF2 fusion protein as substrate. Treatment with TGFβ increased γ-^{32}P incorporation into GST-ATF2 in a time-dependent fashion, sixfold at 15 minutes and reaching a maximal stimulation of 24-fold above control by 60 minutes (Fig. 3B). This increase in p38MAPK kinase activity at 15 minutes may reflect a higher sensitivity of the in vitro kinase assay compared with detection of phosphorylated p38MAPK by immunoblot. TGFβ-induced activation of p38MAPK was dose-dependent with 0.1 ng/ml being sufficient to induce phosphorylation of p38MAPK with a maximal effect observed between 0.5 and 2 ng/ml (Fig. 3C). Treatment of SiHa human cervical carcinoma cells with TGFβ1 for 60 minutes resulted in phosphorylation of p38MAPK (Fig. 3D), suggesting activation of p38MAPK signaling in response to TGFβ1 in these cells.

Kinase activities of TGFβ receptors are required for TGFβ-induced p38MAPK activation

To confirm the role of TGFβ receptors in activation of p38MAPK, we engineered cells expressing TβRII-K277R, a kinase-inactive mutant of TGFβ type II receptor (Wrana et al., 1994). NMuMG cells were infected with retrovirus encoding TβRII-K277R and enhanced green fluorescent protein (EGFP) or with control retrovirus encoding EGFP only (Gabe). Fluorescent cells were selected by flow cytometry and expression of the HA-tagged mutant receptor was confirmed by immunoblot analysis (Fig. 4A). TGFβ-mediated phosphorylation of Smad2, MKK3/6, and p38MAPK was inhibited in TβRII-K277R cells compared with control Gabe cells (Fig. 4B). TβRII-K277R also blocked EMT (Fig. 4D) and cell migration (Fig. 8A), indicating that TβRII kinase activity is required for these TGFβ responses.

To determine whether the activation of p38MAPK was TGFβ-specific, we expressed wild-type TβRI/Alk5 (Alk5-WT), kinase-inactive Alk5-K232R, or kinase active Alk5-

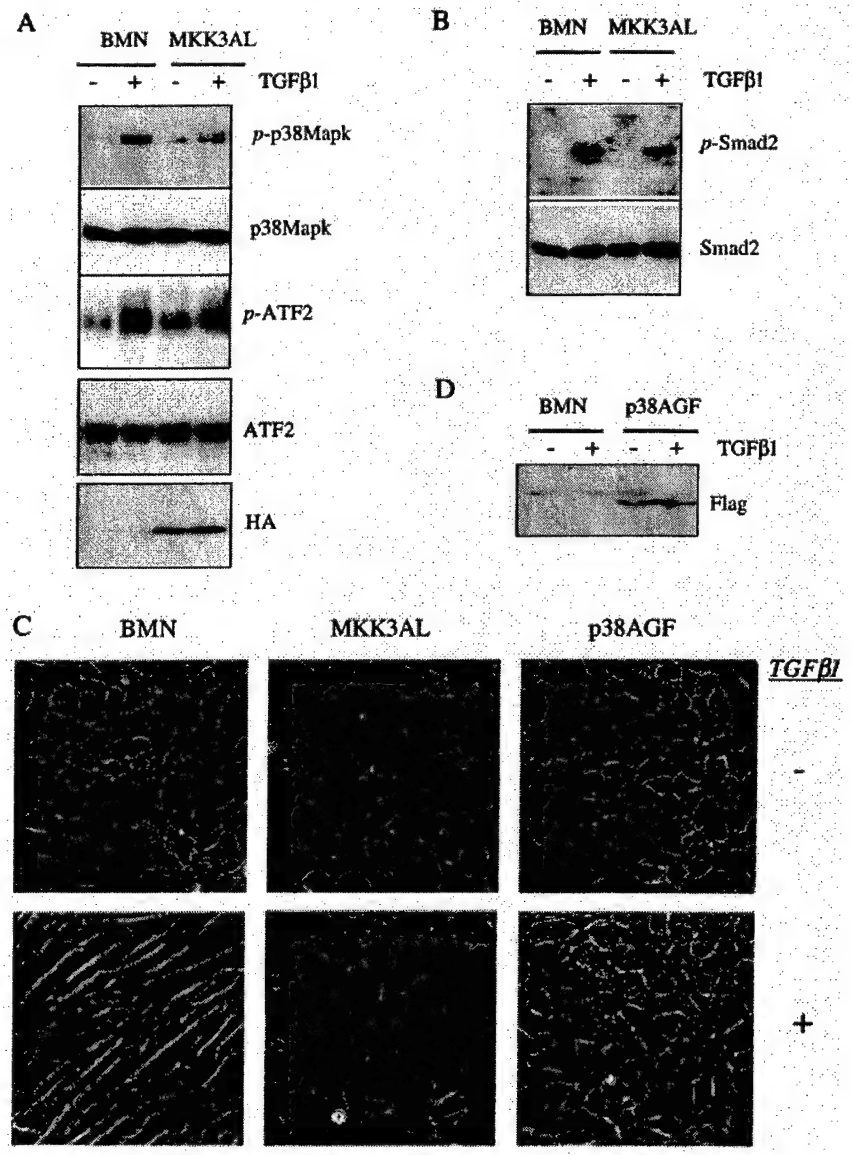

Fig. 5. Effect of dominant-negative MKK3AL and p38AGF on TGFβ-mediated EMT. (A) Immunoblot analysis of p38MAPK and ATF2 phosphorylation in NMuMG cells transfected with empty vector (BMN) or plasmid encoding HA-tagged MKK3AL. Thirty-six hours after transfection cells were treated with 2 ng/ml TGFβ1 for 60 minutes. Whole-cell extracts were probed with phospho-specific antisera, and re-probed with antisera to total protein. Expression of MKK3AL was detected with antisera to the HA-epitope. (B) Immunoblot detection of Smad2 phosphorylation in MKK3AL-expressing cells. (C) Phase-contrast images of NMuMG cells infected with control (BMN) retrovirus or retroviruses encoding dominant-negative MKK3 (MKK3AL) or p38α (p38AGF). Cells were untreated (top row) or treated with 2 ng/ml TGFβ1 for 24 hours. Images were recorded at 200× magnification. (D) Immunoblot detection of Flag-tagged p38AGF in NMuMG cells infected with p38AGF encoding retrovirus compared with control retrovirus (BMN).

T204D (Kawabata et al., 1995) in NMuMG cells. Alk5 mutants were expressed using a bi-cistronic retroviral vector encoding EGFP. GFP-positive cells were selected by flow cytometry and expression of mutants was confirmed by immunoblot analysis (Fig. 4B). Kinase-inactive Alk5-K232R significantly reduced TGFβ-induced phosphorylation of MKK3/6 and p38MAPK, whereas kinase active Alk5-T204D induced phosphorylation of MKK3/6 and p38MAPK in the absence of added ligand (Fig. 4B). Microscopic studies showed that TGFβ-induced EMT was impaired in cells expressing kinase-inactive Alk5-K232R. Cells expressing Alk5-T204D exhibited a fibroblastic morphology similar to Alk5-WT cells treated with TGFβ for 24 hours (Fig. 4C). Treatment of cells expressing Alk5-T204D with the p38MAPK inhibitor SB202190 reversed these morphological changes.

MKK3/6 kinases mediate activation of p38MAPK and EMT in response to TGFβ

Dual-specificity MKK3 and MKK6 kinases have been implicated in activation of p38MAPK (Raingeaud et al., 1996). Phosphorylation of both kinases is induced by TGFβ or by expression of active Alk5-T204D in NMuMG cells (Fig. 4B). Therefore, we tested the effect of dominant-negative MKK3AL (Huang et al., 1997; Zanke et al., 1996) on TGFβ-mediated activation of p38MAPK and EMT in NMuMG cells. Expression of HA-tagged MKK3AL reduced phosphorylation of endogenous p38MAPK and ATF2 (Fig. 5A), whereas expression and phosphorylation of Smad2 were not affected (Fig. 5B). Similar results were obtained with dominant-negative MKK6AL (data not shown). Next, we examined the effect of MKK3AL on EMT. TGFβ induced EMT in NMuMG cells infected with control retrovirus encoding EGFP only (BMN), whereas EMT was inhibited in MKK3AL-expressing cells (Fig. 5C). SB202190, a p38MAPK inhibitor, blocks activity of p38α and p38β but does not inhibit p38γ and p38δ (Davies et al., 2000). Since, SB202190 blocked EMT (Fig. 2A), we tested the effect of p38AGF, a dominant-negative mutant of p38α, on TGFβ-mediated EMT. TGFβ-induced morphological transformation in NMuMG infected with retroviruses encoding p38AGF was impaired compared with cells infected with control BMN virus (Fig. 5C). These findings suggest that MKK3/6 kinases mediate TGFβ-induced activation of p38MAPK and EMT in NMuMG cells.

p38MAPK is involved in TGFβ-induced reorganization of the actin cytoskeleton

We characterized the effect of p38MAPK inhibitors on reorganization of the actin cytoskeleton in response to TGFβ. Microscopic examination of F-actin by staining with phalloidin-fluorescein showed a cortical arrangement of actin at the cell-cell junctions without significant stress fibers (Fig. 6A). Treatment with TGFβ1 for 24 hours induced formation of actin stress fibers arranged along the largest cell axis. SB202190 did not significantly change the actin organization in TGFβ-untreated cells, but impaired TGFβ-induced formation of actin stress fibers (Fig. 6A). Similar blockade of stress fiber formation was observed in cells pretreated with H-7 (data not shown). Examination of the actin cytoskeleton in MKK3AL cells showed that MKK3AL did not affect the cortical arrangement of actin in untreated cells, but inhibited TGFβ-induced actin stress fiber formation (Fig. 6B). These

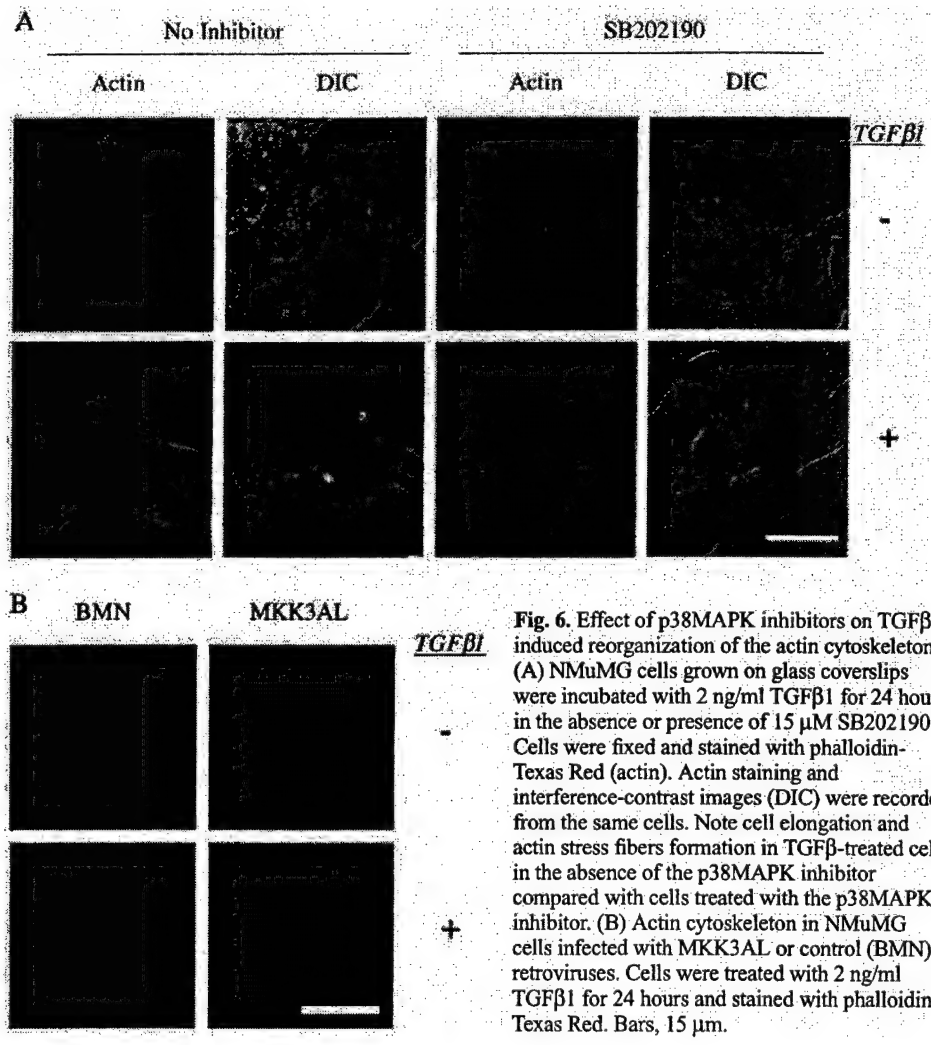

Fig. 6. Effect of p38MAPK inhibitors on TGFβ-induced reorganization of the actin cytoskeleton. (A) NMuMG cells grown on glass coverslips were incubated with 2 ng/ml TGFβ1 for 24 hours in the absence or presence of 15 μM SB202190. Cells were fixed and stained with phalloidin-Texas Red (actin). Actin staining and interference-contrast images (DIC) were recorded from the same cells. Note cell elongation and actin stress fibers formation in TGFβ-treated cells in the absence of the p38MAPK inhibitor compared with cells treated with the p38MAPK inhibitor. (B) Actin cytoskeleton in NMuMG cells infected with MKK3AL or control (BMN) retroviruses. Cells were treated with 2 ng/ml TGFβ1 for 24 hours and stained with phalloidin-Texas Red. Bars, 15 μm.

data suggest that p38MAPK contributes to the reorganization of the actin cytoskeleton induced by TGFβ during EMT.

Rac GTP-binding protein is involved in TGFβ-induced activation of p38MAPK and EMT

There is evidence that small GTP-binding proteins are involved in TGFβ signaling (Atfi et al., 1997; Bakin et al., 2000; Bhowmick et al., 2001a; Engel et al., 1999; Mucsi et al., 1996). Rac1 and CDC42 have been implicated in the activation of the MKK3/6-p38MAPK cascade in several systems (Coghlan et al., 2000; Tibbles et al., 1996; Uddin et al., 2000; Zhang et al., 1995). To test whether Rac1 or RhoA are involved in p38MAPK activation in response to TGFβ, we transfected dominant-negative RhoAN19 or Rac1N17 in NMuMG cells. Rac1N17 inhibited TGFβ1-induced phosphorylation of p38MAPK and its downstream substrate ATF2, whereas neither dominant-negative RhoAN19 nor constitutively active RhoAQ63L did not affect p38MAPK phosphorylation (Fig. 7A,B). These data suggest that Rac1 mediates p38MAPK activation in response to TGFβ.

To examine whether Rac1 activity is induced by TGFβ, we performed affinity precipitation assays using a fusion protein of the GTPase-binding domain (amino acids 67-152) from human PAK1 (PBD) and GST. The GST-PBD fusion protein has been shown to specifically bind active Rac1 loaded with GTP (Benard et al., 1999). Treatment of NMuMG cells for 15 minutes with TGFβ resulted in the increase in Rac1 binding to purified GST-PBD (Fig. 7C). GST-PBD effectively interacted with the active GTPγS-bound form of Rac1 but did not bind to the inactive GDP-bound form of Rac1 (Fig. 7C, left inset). To confirm that TGFβ receptors can mediate activation of Rac1, we expressed mutants of Alk5-TβRI in HEK293T cells. Kinase-inactive Alk5K232R reduced the level of active Rac1, whereas kinase-active Alk5T204D increased the amount of Rac1 bound to GST-PBD. Expression of dominant-negative Rac1N17 reduced the amount of Rac1 recovered from GST-PBD beads (Fig. 7D). Since active Rac1

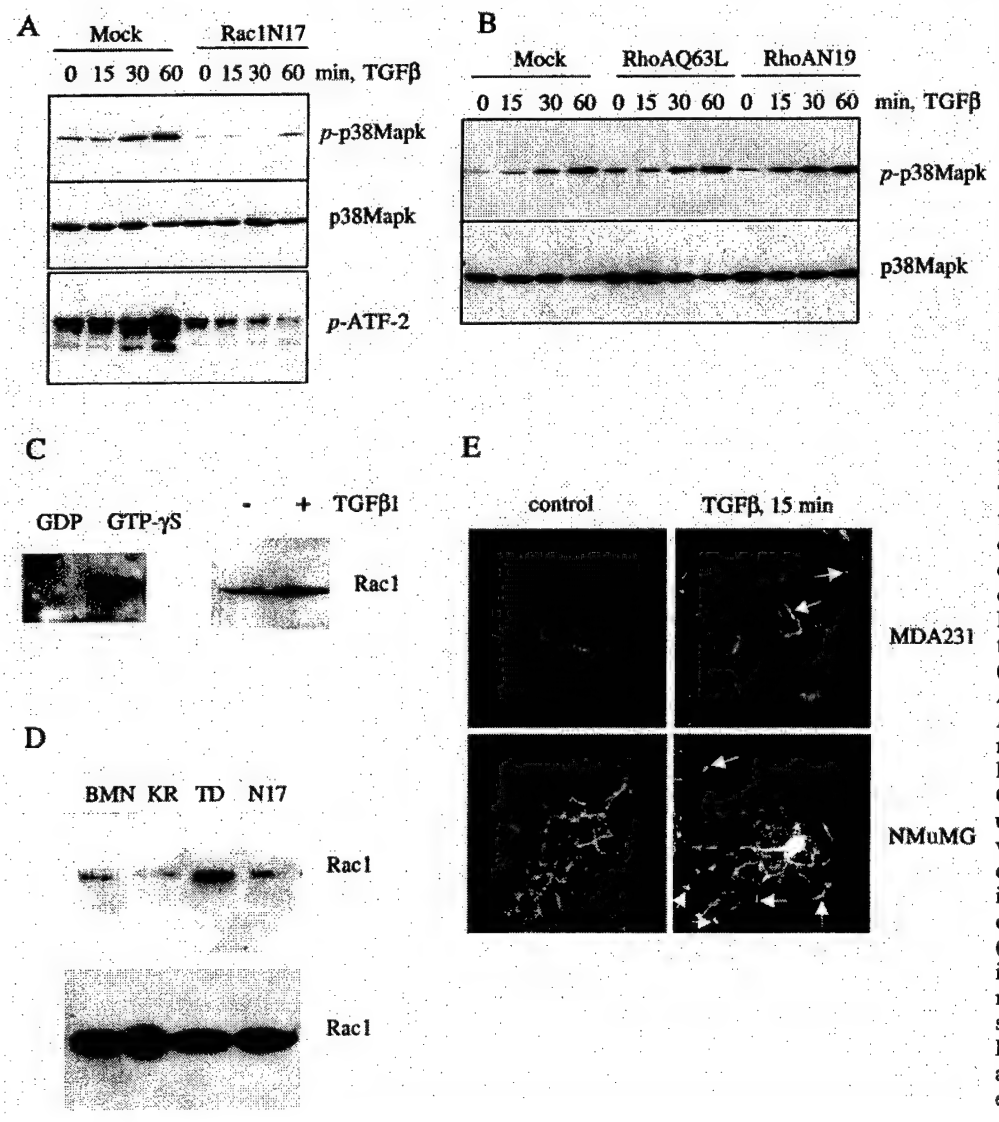

Fig. 7. Rac1 is involved in TGFβ-mediated activation of p38MAPK. (A) Immunoblot analysis of p38MAPK and ATF2 phosphorylation in cells expressing Rac1N17 and treated with 2 ng/ml TGFβ1. (B) p38MAPK phosphorylation in cells expressing RhoAN19 or RhoAQ63L. (C) NMuMG cells were treated with 2 ng/ml TGFβ1 for 15 minutes. Cell lysates were clarified and used for affinity precipitation with 8 μg of GST-PBD. Proteins bound to GST-PBD were separated on SDS-PAGE, transferred to nitrocellulose membrane and blotted with antibody to Rac1. The inset at the top-left shows the total signal detected using cell lysate pre-exchanged with either GTPγS or GDP as described in Materials and Methods. (D) 293T cells were transfected with control plasmid (BMN), kinase-inactive Alk5K232R, kinase-active Alk5T204D or dominant-negative Rac1N17. Cells were lysed 48 hours after transfection. Cell lysates were clarified and used for affinity precipitation with 8 μg of GST-PBD as described above. The bottom inset shows the Rac1 signal detected in total cell lysates. (E) Confocal images of F-actin in NMuMG cells treated with 2 ng/ml TGFβ1 for 15 minutes and stained with phalloidin-Texas Red. Arrows indicate the spots of actin polymerization at the cell edges.

mediates actin ruffling and lamellipodia formation (Hall, 1998), we examined F-actin in NMuMG and MDA-MB-231 cells treated with 2 ng/ml of TGFβ1 for 15 minutes. Confocal microscopy of cells stained with phalloidin-Texas Red showed that TGFβ induced actin ruffles, a phenotype associated with active Rac (Fig. 7E).

In order to examine the role of Rac1 in EMT, NMuMG cells were infected with a retrovirus encoding dominant-negative Rac1N17 and Green Fluorescent Protein (GFP). Immunoblot analysis showed at least twofold higher levels of Rac1 in cells infected with Rac1N17 retrovirus compared with cells infected with control BMN virus encoding GFP only (Fig. 8A). TGFβ induced phosphorylation of MKK3/6 and p38MAPK in cells infected with control retrovirus whereas, in Rac1N17 cells, this induction was significantly reduced (Fig. 8B). Rac1N17 did not significantly affect TGFβ-dependent phosphorylation of Smad2 (Fig. 8C). Microscopic examination showed that TGFβ1 induced cell elongation and the formation of actin stress fibers in control BMN cells, whereas these effects were impaired in cells expressing Rac1N17 (Fig. 8D). These findings suggest that Rac1 is involved in TGFβ-induced EMT and activation of p38MAPK.

p38MAPK inhibitors block TGFβ-mediated cell motility

TGFβ stimulates chemotaxis and migration of tumor and nontumor cells (Ashcroft et al., 1999; Postlethwaite et al., 1987). Recent studies implicated p38MAPK in TGFβ-induced chemotaxis of human neutrophils (Hannigan et al., 1998). We next tested the effect of p38MAPK inhibitors on TGFβ-mediated migration of NMuMG (nontumor) and MDA-MB-231 (tumor) cells. TGFβ stimulated approximately threefold the chemotactic migration of NMuMG cells through polycarbonate filters (Fig. 9A). Migration of NMuMG cells was significantly inhibited by SB202190, as were NMuMG cells infected with kinase-inactive TGFβ type II receptor (TβRII-K277R) compared with those infected with control Gabe retrovirus (Fig. 9A). TGFβ stimulated approximately sixfold migration of breast cancer MDA-MB-231 cells. This was also blocked by SB202190 (Fig. 9B).

To investigate further the role of p38MAPK in TGFβ-mediated cell migration, wounds were made in confluent cultures of MDA-MB-231 and 4T1 breast cancer cells. These cells are not growth inhibited by TGFβ1. Addition of TGFβ1 to serum-free medium accelerated the wound closure in both cell lines, whereas in the presence of the p38MAPK inhibitor

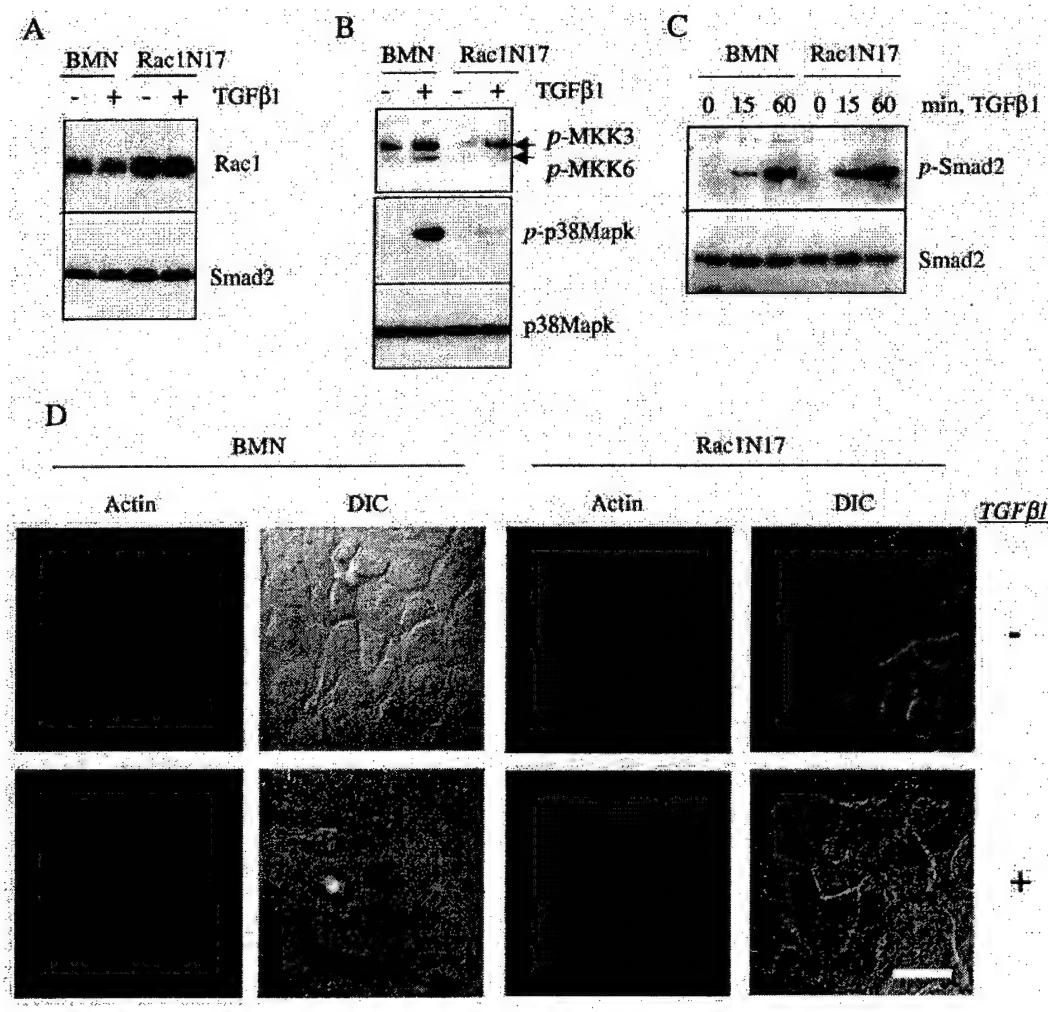

Fig. 8. Dominant-negative Rac1N17 blocks TGFβ-mediated activation of p38MAPK and EMT. NMuMG cells were infected with retrovirus encoding Rac1N17 or control virus (BMN) and treated with 2 ng/ml TGFβ1. (A) Detection of Rac1N17 expression with antisera to Rac1. Cells infected with Rac1N17 show a higher level of Rac1 expression. (B) Immunoblot detection of MKK3/6 and p38MAPK phosphorylation in control (BMN) and Rac1N17 expressing cells. (C) Immunoblot with antisera to phospho-Smad2 and total Smad2. (D) Microscopic images from NMuMG cells infected with control retrovirus (BMN) or retrovirus encoding Rac1N17. Cells grown on glass coverslips were treated with 2 ng/ml TGFβ1 for 24 hours. Cells were stained with phalloidin-Texas Red (actin). Actin and interference-contrast images (DIC) were recorded from the same cells. Bar, 15 μm.

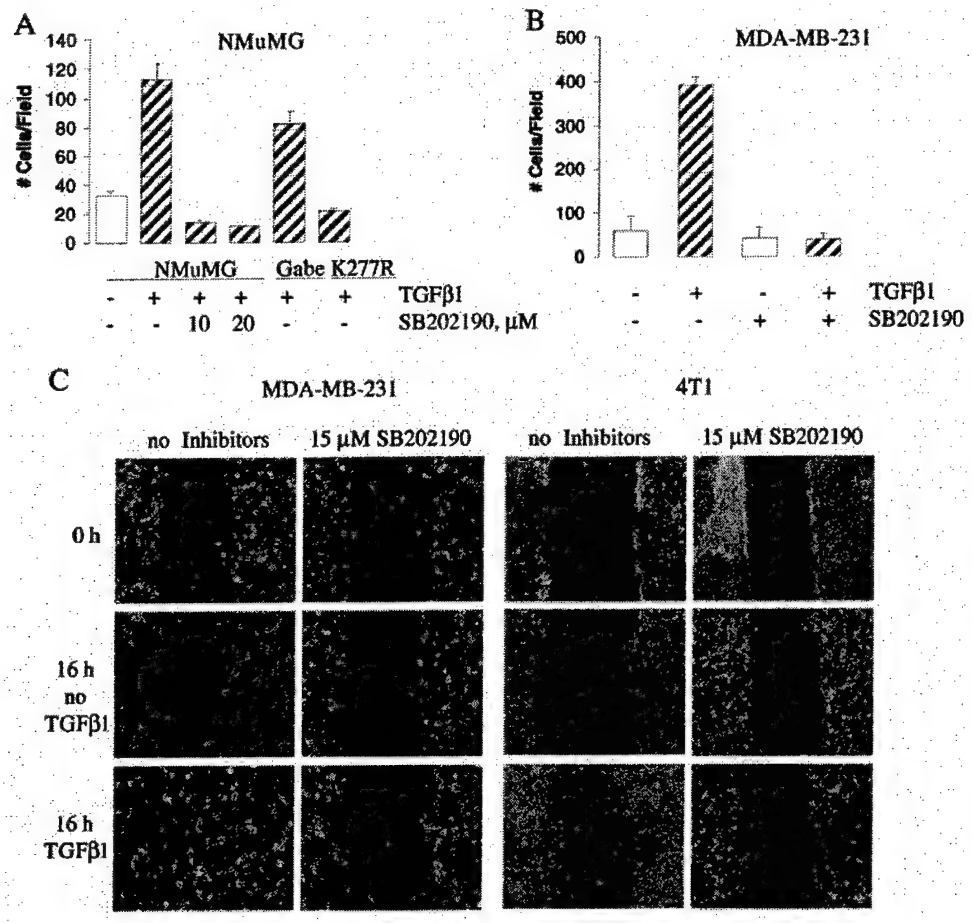

Fig. 9. Involvement of p38MAPK in TGFβ-mediated cell migration. (A,B) NMuMG or MDA-MB-231 cells (1×10^5/well) were seeded in the upper chamber of 5 μm pore transwells and 2 ng/ml TGFβ1 was added to the lower chamber. Cells were incubated for 16 hours in the absence or presence of SB202190, a p38MAPK inhibitor. Cells migrating through pores were stained and counted from four random fields. Experiments were performed in duplicates. Values are the mean ±s.d. of cells per field. Migration of NMuMG cells expressing kinase-inactive TβRII-K277R was compared with cells infected with control Gabe virus. (B) Blockade of MDA-MB-231 cell migration with 10 μM SB202190. (C) Wound closure in monolayers of MDA-MB-231 and 4T1 cells following 16 hours of treatment with 2 ng/ml TGFβ1 in the absence or presence of 10 μM SB202190. Phase contrast images were recorded at 100× magnification. Similar results were obtained three times.

the wounds stayed opened (Fig. 9D). These data suggest that p38MAPK is involved in TGFβ-induced cell migration.

Discussion

TGFβ can induce mesenchymal transdifferentiation in epithelial and endothelial cells (Boyer et al., 1999; Brown et al., 1999; Miettinen et al., 1994). Early studies have shown that protein kinase inhibitor H-7 blocks TGFβ-induced EMT but a particular signaling cascade affected by H-7 was not identified (Miettinen et al., 1994). We found that H-7 inhibited TGFβ-induced phosphorylation of MKK3/6 kinases, but did not affect phosphorylation of Smad2 and Smad-dependent transcriptional responses. These results suggest that H-7 affects a kinase that mediates signaling downstream of TGFβ receptors, but upstream of MKK3/6 kinases. This kinase is distinct from typical PKCs, since BIM-I, an inhibitor of typical PKCs, did not block TGFβ-induced EMT and phosphorylation of MKK3/6 and p38MAPK. The candidate kinases include atypical PKCs and kinase(s) implicated in activation of MKK3/6 such as PAK1 (Zhang et al., 1995), TAK1 (Yamaguchi et al., 1995), and MLK3 (Tibbles et al., 1996).

The H-7 studies suggested a critical role for the p38MAPK pathway in EMT. This hypothesis was further tested using the p38MAPK specific inhibitors, SB202190 and SB203580, which do not inhibit JNK, MEK1/2 and ERK1/2 kinases (Davies et al., 2000). SB202190 and SB203580 blocked TGFβ-induced cell morphological changes in NMuMG mouse mammary epithelial cells and SiHa human cervical carcinoma cells. The p38MAPK inhibitors blocked TGFβ-induced phosphorylation of ATF2, a p38MAPK substrate, without effect on Smad2 phosphorylation, implying that under these experimental conditions the blockade of p38MAPK did not affect TGFβ receptor kinase activity.

To test whether activation of p38MAPK by TGFβ is a direct event, we investigated the kinetics of activating phosphorylation of MKK3/6 and p38MAPK. TGFβ induced phosphorylation of Smad2 and MKK3/6 kinases with similar kinetics (15 minutes). Phosphorylation of p38MAPK was delayed (30 minutes) suggesting that this event requires activation of MKK3/6. We further showed that dominant-negative mutants of MKK3 and MKK6 interfering with p38MAPK activation (Raingeaud et al., 1996) impaired TGFβ-induced phosphorylation of p38MAPK and ATF2, indicating that the MKK3/6-p38MAPK module mediates TGFβ signaling in NMuMG cells. The dose-dependent increase in p38MAPK activity was confirmed by in vitro kinase assay and by phosphorylation of ATF2.

To confirm the specificity of TGFβ signaling to p38MAPK we performed studies with TGFβ receptor mutants. Kinase-inactive type II receptor blocked EMT and phosphorylation of Smad2 as well as MKK3/6 and p38MAPK, indicating that

kinase function of TβRII is required for activation of p38MAPK and EMT. Kinase-inactive TβRI/Alk5-K232R also blocked TGFβ-induced activation of the p38MAPK pathway, whereas expression of kinase active Alk5-T204D resulted in phosphorylation of MKK3/6 and p38MAPK and EMT in the absence of added TGFβ1. Thus, kinase activities of both TGFβ receptors are required for TGFβ-induced activation of the p38MAPK pathway, and Alk5-T204D can signal to p38MAPK in the absence of added ligand. Alk5-T204D-induced EMT was inhibited by SB202190, a p38MAPK inhibitor, suggesting that p38MAPK mediates EMT induced by Alk5-T204D.

Activation of p38MAPK is mediated by Rac1/CDC42 GTP-binding proteins (Coghlan et al., 2000; Tibbles et al., 1996; Uddin et al., 2000; Zhang et al., 1995). Small GTP-binding proteins are also involved in TGFβ responses (Atfi et al., 1997; Bakin et al., 2000; Bhowmick et al., 2001a; Engel et al., 1999; Mucsi et al., 1996). We found that dominant-negative Rac1N17 impaired activation of the p38MAPK pathway in NMuMG cells, whereas RhoAN19 did not block this event. Expression of Rac1N17 did not affect phosphorylation of Smad2. These data suggest that Rac1 mediates TGFβ-induced p38MAPK activation independently of Smad activation. The mechanism of downstream signaling events is unclear. Previous studies showed that PAK1 mediates p38MAPK activation downstream of Rac1 and CDC42 (Zhang et al., 1995). Furthermore, TGFβ-activated kinase 1 (TAK1), has been implicated in p38MAPK activation in response to BMP and TGFβ in several cell systems (Yamaguchi et al., 1995).

Expression of dominant-negative Rac1N17 in NMuMG cells inhibited TGFβ1-induced changes in cell shape and the actin cytoskeleton suggesting involvement of Rac1 in TGFβ-induced EMT. This result is consistent with other reports. For example, both D-Rac and D-p38 have been reported to contribute to Dpp signaling during wing morphogenesis in *Drosophila* (Adachi-Yamada et al., 1999; Eaton et al., 1995). There is also evidence that Rac1 is required for EMT induced by hepatocyte growth factor (HGF) in MDCK cells (Ridley et al., 1995; Royal et al., 2000). Dominant-negative Rac/CDC42 mutants inhibit oncogenic Ras-induced cell transformation (Qiu et al., 1997; Qiu et al., 1995), and Ras has been shown to cooperate with TGFβ in the induction of EMT (Oft et al., 1996). In addition, Rho/Rac/CDC42 proteins are involved in morphogenesis by regulating the actin cytoskeleton (Hall, 1998). Therefore, Rac1 may contribute to TGFβ-induced EMT via its effects on the cell cytoskeleton and/or via activation of the p38MAPK pathway. In NMuMG cells, TGFβ1 induced actin ruffles and activation of Rac1 within 15 minutes (Fig. 7C,E). Expression of kinase-inactive Alk5K232R reduced, whereas constitutively active Alk5-T204D increased, Rac1 loading with GTP (Fig. 7D) and induced the formation of strong actin ruffles (data not shown). These results suggest that Rac activation and actin ruffling induced by TGFβ may precede the formation of actin stress fibers, which does not occur until 4 hours after addition of TGFβ1 (Bhowmick et al., 2001a).

Inhibitors of p38MAPK and dominant-negative MKK3AL impaired TGFβ-induced changes in cell morphology and reorganization of the actin cytoskeleton. Expression of the dominant-negative mutant of p38α also blocked TGFβ-mediated EMT. Together, these results suggest that the p38MAPK pathway contributes to TGFβ-induced alterations in the actin cytoskeleton and the cell shape during EMT. Consistent with this hypothesis, p38MAPK has been shown to mediate regulation of the actin cytoskeleton in smooth muscle myocytes in response to TGFβ (Hedges et al., 1999), and in H_2O_2-induced rapid reorganization of the actin cytoskeleton in endothelial and mesenchymal cells (Huot et al., 1998). A recent study reported involvement of p38MAPK in TGFβ-mediated EMT (Bhowmick et al., 2001b). In this report, adenoviral transduction of dominant-negative p38β inhibited TGFβ-mediated EMT at the step of disruption of junctional complexes but did not alter actin reorganization. We found that p38MAPK inhibitors and dominant-negative MKK3AL affected actin stress fiber formation (Fig. 6). TGFβ and Alk5T204D activated both MKK3 and MKK6 in NMuMG cells (Figs 3, 4). This suggests that TGFβ may activate multiple p38MAPK isoforms in NMuMG cells as MKK3 preferentially activates p38α and p38γ, while MKK6 activates p38MAPKs α, β and γ (Enslen et al., 1998). Recent studies showed that p38α and p38β may have different functions (Wang et al., 1998) and different subcellular localization (Lee et al., 2000). p38MAPK inhibitors block activity of both p38α and p38β (Enslen et al., 1998) and MKK3AL impaired phosophorylation of p38MAPK in NMuMG cells as measured with an antibody that recognizes both α and β isoforms. Therefore, multiple p38MAPKs may be involved in TGFβ-induced EMT and mediate different aspects of EMT, potentially explaining the discrepancies with previous studies (Bhowmick et al., 2001b).

EMT is a complex process involving restructuring of the cell cytoskeleton, cell membrane and cell-cell junctions. Previous studies implicated several molecules in different aspects of EMT. Smad transcription factors have been shown to synergize with Alk5 in induction of EMT but no specific function has been associated with these factors (Piek et al., 1999a). PI3K/Akt may contribute to dissolution of tight junctions and to TGFβ transcriptional responses (Bakin et al., 2000). RhoA/Rock signaling has been implicated in the actin stress fiber formation (Bhowmick et al., 2001a). What aspect of EMT can be mediated by p38MAPK? p38MAPK can regulate the actin organization via HSP27 (Hedges et al., 1999; Huot et al., 1998). Therefore, p38MAPK may function in TGFβ-induced reorganization of the actin cytoskeleton in parallel or upstream of the RhoA/Rock pathway since dn-RhoA and Y27632, a Rock kinase inhibitor, did not affect activation of p38MAPK by TGFβ (data not shown). In addition, p38MAPK may contribute to the expression of TGFβ target genes that are casually involved in EMT because p38MAPK has been implicated in TGFβ-transcriptional responses by activating ATF2 and Sp1 (Park et al., 2000; Raingeaud et al., 1996; Sano et al., 1999).

Finally, we investigated the role of p38MAPK in TGFβ-induced migration of mouse and human mammary epithelial cells. The p38MAPK inhibitors blocked TGFβ-stimulated migration of NMuMG, MDA-MB-231 and 4T1 cells. These results are consistent with the proposed role of p38MAPK in TGFβ-mediated chemotaxis of human neutrophils (Hannigan et al., 1998) and smooth muscle cells (Hedges et al., 1999). Interestingly, Smad3-deficient keratinocytes and monocytes are impaired in the chemotactic response to TGFβ (Ashcroft et al., 1999), whereas p38MAPK inhibitors did not affect Smad2 phosphorylation (Fig. 2). These data suggest that the p38MAPK pathway may act in parallel or in cooperation with a Smad-dependent pathway in chemotactic responses to TGFβ.

The data presented suggest that p38MAPK signaling plays a critical role in TGFβ-induced EMT and cell migration. This pathway may be considered as a potential target of therapeutic interventions in neoplastic and inflammatory disorders associated with TGFβ-mediated EMT.

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Autocrine Transforming Growth Factor-β Signaling Mediates Smad-independent Motility in Human Cancer Cells*

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Transforming growth factor-β (TGF-β) is a pleiotropic growth factor that plays a critical role in modulating cell growth, differentiation, and plasticity. There is increasing evidence that after cells lose their sensitivity to TGF-β-mediated growth inhibition, autocrine TGF-β signaling may potentially promote tumor cell motility and invasiveness. To understand the molecular mechanisms by which autocrine TGF-β may selectively contribute to tumor cell motility, we have generated MDA-MB-231 breast cancer cells stably expressing a kinase-inactive type II TGF-β receptor (TβRII-K277R). Our data indicate that TβRII-K277R is expressed, can associate with the type I TGF-β receptor, and block both Smad-dependent and -independent signaling pathways activated by TGF-β. In addition, wound closure and transwell migration assays indicated that the basal migratory potential of TβRII-K277R expressing cells was impaired. The impaired motility of TβRII-K277R cells could be restored by reconstituting TGF-β signaling with a constitutively active TGF-β type I receptor ($ALK5^{TD}$) but not by reconstituting Smad signaling with Smad2/4 or Smad3/4 expression. In addition, the levels of $ALK5^{TD}$ expression sufficient to restore motility in the cells expressing TβRII-K277R were associated with an increase in phosphorylation of Akt and extracellular signal-regulated kinase 1/2 but not Smad2. These data indicate that different signaling pathways require different thresholds of TGF-β activation and suggest that TGF-β promotes motility through mechanisms independent of Smad signaling, possibly involving activation of the phosphatidylinositol 3-kinase/Akt and/or mitogen-activated protein kinase pathways.

Transforming growth factor-β (TGF-β)[1] is a pleiotropic polypeptide growth factor that is part of a superfamily of structurally related ligands that includes the TGF-βs, activins, and bone morphogenetic proteins (BMPs) (1). TGF-β ligands play a critical role in modulating cell growth, differentiation, plasticity, and migration. They elicit their biological effects by binding to a heteromeric complex of transmembrane serine/threonine kinases, the type I and type II receptors. TGF-β ligands can also bind to a transmembrane proteoglycan referred to as the type III receptor, which is thought to present ligand to the signaling type I and type II receptors. Following ligand binding to the type II receptor, the type I receptor is recruited to the complex. This allows the type II receptor, which is a constitutively active kinase, to transphosphorylate and thereby activate the type I receptor (2). Multiple pathways have now been implicated in mediating TGF-β effects downstream of these receptors. These include the extracellular signal-regulated kinase (ERK) (3, 4), c-Jun NH_2-terminal kinase (JNK) (5–7), p38 mitogen-activated protein kinase (MAPK) (8, 9), and phosphatidylinositol 3-kinase (PI3K) pathways (10, 11). Several small GTPases can also be activated by TGF-β (12) and are involved in the activation of many of the above-mentioned signaling pathways. However, the Smad pathway was the first signaling pathway identified to mediate TGF-β effects and remains the best characterized (reviewed in Ref. 1).

Signal transduction through the Smad pathway involves phosphorylation of a set of intracellular signaling proteins termed receptor-regulated Smads (R-Smads) by the activated type I receptor. Once phosphorylated, R-Smads can associate with a common mediator Smad, Smad4, translocate to the nucleus, and regulate gene transcription. In addition to the R-Smads and the common mediator Smad, Smad4, there is a distinct, structurally related class of antagonistic Smads, Smad6 and Smad7, which inhibit TGF-β family signals. Smad6 preferentially inhibits BMP signaling by either competing with Smad4 for binding to R-Smads (13) or interfering with BMP receptor-mediated phosphorylation of Smads (14). Smad7 has been reported to inhibit both TGF-β and BMP signaling by binding to activated type I receptors and interfering with their ability to phosphorylate R-Smads (15, 16).

Although TGF-β1 was originally identified for its ability to cause reversible phenotypic transformation and anchorage-independent growth of fibroblasts (17, 18), TGF-β can act as both a tumor suppressor and a tumor promoter (19, 20). TGF-β elicits most of its tumor suppressor activity by potently inhibiting the proliferation of most epithelial cells. It is thought that escape from the growth inhibitory effects of TGF-β through

* Fluorescence microscopy images were acquired through the use of the Vanderbilt University Medical Center Cell Imaging Core Resource supported by National Institutes of Health Grants CA68485 and DK20593. This work was supported in part by United States Army Medical Research and Materiel Command Awards DAMD17-98-1-8263 (to N. D.), BC011342 (to A. V. B.), and DAMD17-98-1-8262 (to C. L. A.), by Public Health Service Grants CA62212 (to C. L. A.) and CA95263 (to A. V. B.), and by Vanderbilt-Ingram Cancer Center Support Grant CA68485. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: TGF-β, transforming growth factor-β; BMP, bone morphogenetic protein; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; R-Smad, receptor-regulated Smad; dn, dominant-negative; FCS, fetal calf serum; ALK, activin-like receptor kinase; HA, hemagglutinin; GFP, enhanced green fluorescent protein; m.o.i., multiplicity of infection; MEK, MAPK/ERK kinase; D-PBS, Dulbecco's phosphate-buffered saline; EMT, epithelial mesenchymal transformation.

dysregulated expression or mutational inactivation of various components of the TGF-β signaling pathway can contribute to tumorigenesis (21–23). In addition, there is increasing evidence that after cells lose their sensitivity to TGF-β-mediated growth inhibition, autocrine TGF-β signaling may promote tumorigenesis. The importance of autocrine TGF-β signaling in tumor progression has been highlighted by several studies that have shown that expression of a dominant-negative type II TGF-β receptor (dnTβRII) in various tumor cells can prevent the conversion of cells from an epithelial to an invasive mesenchymal phenotype, delay tumor growth, and reduce metastases (24–27). These data suggest that TGF-β can act directly on tumor cells to promote tumor maintenance and progression. In addition to promoting epithelial to mesenchymal transformation of tumor cells, TGF-β can stimulate the motility of many cell types *in vitro*, suggesting that TGF-β production *in vivo* may enhance migration of tumor cells and thus contribute to tumor invasiveness and metastases. There is also evidence that TGF-β can increase cellular motility without affecting proliferation, suggesting that the effects on motility and proliferation may occur via different biochemical pathways (28).

To understand the molecular mechanisms by which autocrine TGF-β may selectively contribute to tumor cell motility, we have generated MDA-MB-231 breast cancer cells stably expressing dnTβRII. MDA-MB-231 cells express TGF-β receptors (29), secrete TGF-β (30), and, although they are resistant to the growth inhibitory effects of TGF-β (29), can respond to TGF-β with an increase in spreading (31) and invasiveness (32). In addition, there is evidence that blocking TGF-β signaling by administration of a neutralizing TGF-β antibody can inhibit MDA-MB-231 cell tumorigenicity and metastases in nude mice (33). In this paper we show that expression of dnTβRII in MDA-MB-231 cells impairs their basal migratory potential. This impairment in motility can be restored by expression of a constitutively active type I TGF-β receptor ($ALK5^{TD}$) but not by overexpression of Smad2/4 or Smad3/4. In addition, the levels of $ALK5^{TD}$ expression sufficient to restore motility in the cells expressing dnTβRII are associated with an increase in phosphorylation of Akt and ERK1/2, but not Smad2, suggesting that Smad signaling is dispensable for autocrine TGF-β-mediated motility and that this response depends on alternative signaling pathways activated by TGF-β.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—The MDA-MB-231 and MDA-MB-468 breast cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA) and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). The SW480.7 clone 15.13 (34) was a gift from Dr. Joan Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY) and was maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 0.3 mg/ml Zeocin, and 0.7 mg/ml G418. TGF-β1 and BMP2 were obtained from R&D Systems (Minneapolis, MN). Peter ten Dijke (The Netherlands Cancer Institute, Amsterdam, The Netherlands) graciously provided the rabbit polyclonal sera directed against activin-like receptor kinases (ALKs) (35). Antibodies against the hemagglutinin (HA) epitope (catalog number sc-7392), the type II TGF-β receptor (catalog number sc-220), Smad4 (catalog number sc-7966), and p38 MAPK (catalog number sc-7972) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to fibronectin (catalog number F14420) and Smad2/3 (catalog number S66220) were from Transduction Laboratories (San Diego, CA). The C-terminal phospho-Smad2 antibody (catalog number 06-829) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). The C-terminal phospho-Smad1 antibody (catalog number 9511) and the phospho-p38 MAPK antibody (catalog number 9211S) were from Cell Signaling Technology (Beverly, MA). Monoclonal antibodies to actin (catalog number A-4700) and the FLAG epitope (catalog number F3165), as well as the polyclonal antibody to α-catenin (catalog number C2081), were obtained from Sigma. Phalloidin-Texas Red and Hoechst 3342 were from Molecular Probes (Eugene, OR). LY294002, SB202190, and JNKiII were purchased from Calbiochem. The MEK inhibitor, UO126, was purchased from Promega (Madison, WI).

Generation of Stable Cell Lines—To generate MDA-MB-231 cells stably expressing dnTβRII, we obtained a construct encoding a kinase-inactive TβRII mutant in which the lysine at position 277 has been mutated to arginine (pGABE-TβRII-K277R) (25) from Martin Oft (UCSF, San Francisco, CA). Lysine 277 corresponds to an invariant lysine found in the ATP-binding site of subdomain II in all protein kinases, and even its substitution with arginine results in loss of kinase activity (36). The pGABE vector is a modified version of the commonly used retroviral vector pBABE in which the puromycin cassette has been replaced by enhanced green fluorescent protein (GFP). In this construct, TβRII-K277R is HA-tagged, and its expression is driven by the viral long terminal repeat, whereas expression of GFP is driven by the SV40 promoter. MDA-MB-231 cells were transfected with the control pGABE vector or the pGABE-TβRII-K277R vector utilizing LipofectAMINE reagent (Invitrogen) according to the manufacturer's instructions. Following transfection, cells expressing GFP were sorted by flow cytometry. Clones were then isolated by sorting individual cells from the >95% positive GFP pool.

Affinity Labeling of Cells with ^{125}I-TGF-β1 and Immunoprecipitation of HA-tagged TβRII-K277R—^{125}I-TGF-β1 was obtained from PerkinElmer Life Sciences. Near confluent MDA-MB-231 cells, as well as clones and pools stably expressing GFP alone (GABE) or GFP and TβRII-K277R (dnTβRII) in 12-well plates, were washed three times over 30 min with 500 μl of ice-cold 0.1% bovine serum albumin dissolved in Dulbecco's phosphate-buffered saline (D-PBS) containing Ca^{2+} and Mg^{2+}. The cells were then affinity labeled with 100 pM ^{125}I-TGF-β1 as described previously (37), with slight modifications. Briefly, after a 3-h incubation with 100 pM ^{125}I-TGF-β1 at 4 °C, the cells were washed with 500 μl of ice-cold D-PBS, and the ligand-receptor complexes were cross-linked with 400 μl of 1 mM bis(sulfosuccinimidyl)suberate (BS^3; Pierce) for 10 min on ice. The cross-linking reaction was stopped with the addition of 100 μl of 500 mM glycine. Cells were washed twice with 500 μl of D-PBS and solubilized with 125 μl of 20 mM Tris buffer, pH 7.4, containing 1% Triton X-100, 10% glycerol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin, and 2 μg/ml soybean trypsin inhibitor. Solubilized material was centrifuged for 10 min at 4 °C to pellet cell debris. The supernatants were transferred to one-fifth volume of 5× electrophoresis sample buffer, boiled, and vortexed. All samples were analyzed using 3–12% SDS-PAGE and visualized by autoradiography. For immunoprecipitation experiments, the radiolabeled cell lysate from a T75 flask was centrifuged at 5000 × *g*, and the supernatant was split into eight equal aliquots and incubated with antibodies directed against ALKs 1, 2, and 5, the type II TGF-β receptor, or HA overnight at 4 °C. Aliquots of radiolabeled cell lysates incubated with normal rabbit serum or no antibody were used as controls.

Immunoblot Analysis—Cells were washed twice with ice-cold D-PBS and lysed with 50 mM Tris, 150 mM NaCl buffer containing 1% Nonidet P-40, 0.25% deoxycholate, 1 mM EDTA, 20 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin, and 2 μg/ml soybean trypsin inhibitor. Protein content was quantitated utilizing the BCA protein assay reagent (Pierce). Protein extracts were separated by 7.5% or 10% SDS-PAGE and transferred to nitrocellulose membranes at 100 volts for 2 h. Membranes were blocked with 5% nonfat dry milk in TBS-T (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20 (v/v)) for 1 h at room temperature and incubated with primary antibodies diluted in TBS-T plus 2.5% nonfat dry milk over night at 4 °C. The membranes were then washed four times for 10 min with TBS-T, incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature, and rewashed four times for 10 min with TBS-T. Immunoreactive bands were visualized by chemiluminescence (Pierce).

Immunofluorescence—Cells grown on glass coverslips (22 × 22 mm) in 35-mm wells were washed twice with D-PBS, fixed with 4% paraformaldehyde in D-PBS for 15 min, permeabilized with 0.1% Triton X-100 for 10 min, and blocked with 3% nonfat dry milk in D-PBS for 60 min, all at room temperature. Cells were then incubated with primary mouse monoclonal antibodies diluted in 1% nonfat dry milk/D-PBS for 1 h at room temperature and washed three times with D-PBS, followed by incubation with a CY3-conjugated anti-mouse antibody in 1% nonfat dry milk/PBS for an additional hour at room temperature. In some experiments, cell nuclei were stained with 1 μg/ml Hoechst for 10 min at room temperature. After three 10-min washes with D-PBS, coverslips were mounted onto 25 × 75-mm microslides using AquaPolyMount (Polysciences Inc., Warrington, PA). Fluorescent images were captured using a Princeton Instruments cooled CCD digital camera from a Zeiss Axiophot upright microscope.

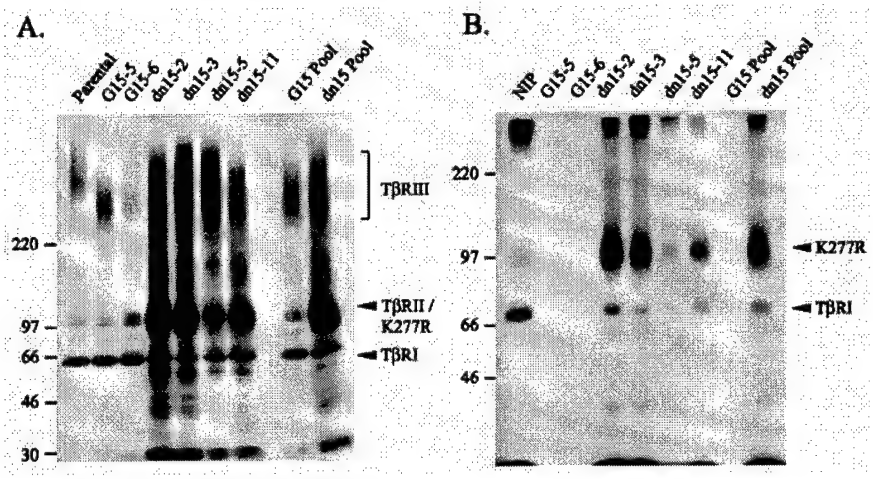

FIG. 1. **TβRII-K277R is expressed in MDA-MB-231 cells.** MDA-MB-231 parental cells, as well as clones and pools stably expressing GFP alone (*G15–5*, *G15–6*, and *G15 Pool*) or GFP and TβRII-K277R (*dn15–2*, *dn15–3*, *dn15–5*, and *dn15–11* clones and *dn15 Pool*), were affinity labeled with 100 pM ^{125}I-TGF-β1 and cross-linked with BS^3. Labeled ligand-receptor complexes were resolved by SDS-PAGE using a 3–12% gradient gel and visualized by autoradiography (*A*) or lysed and incubated with a mouse monoclonal anti-HA antibody for immunoprecipitation of HA-tagged TβRII-K277R (*B*). Immunoprecipitates were resolved by SDS-PAGE using a 7.5% polyacrylamide gel and visualized by autoradiography. Affinity labeled but non-immunoprecipitated (*NIP*) G15–5 cells were loaded as a reference (*lane 1*).

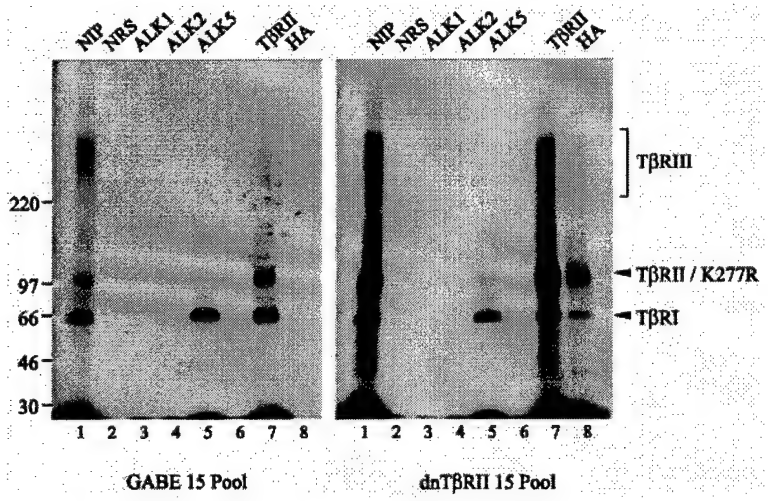

FIG. 2. **TβRII-K277R can associate with TβRI.** MDA-MB-231 pools expressing GFP alone (*GABE 15 Pool*; *left panel*) or GFP and TβRII-K277R (*dnTβRII 15 Pool*; *right panel*) were affinity labeled with 100 pM ^{125}I-TGF-β1, cross-linked with BS^3, lysed, and incubated with normal rabbit serum (*NRS*), polyclonal rabbit antisera directed against various type I TGF-β superfamily receptors (*ALK1*, *ALK2*, and *ALK5*), the type II TGF-β receptor (*TβRII*), or HA as indicated. Immunoprecipitates were resolved by SDS-PAGE using a 3–12% polyacrylamide gel and visualized by autoradiography. Affinity labeled but non-immunoprecipitated (*NIP*) cells were loaded as a reference (*lane 1*).

Transcription Reporter Assays—Cells were transiently transfected with 1 μg per 35-mm dish of the Smad-dependent heterologous promoter reporter construct p(CAGA)$_{12}$-Luciferase (38) provided by Dr. Jean-Michel Gauthier (Laboratoire Glaxo Wellcome, Les Ulis Cedex, France) along with 0.01 μg per 35-mm dish of pCMV-Renilla using FuGENE 6 reagent (Roche Molecular Biochemicals) according to the manufacturer's protocol. The following day, cells were split into 24-well plates, and ~45 h post-transfection, cells were either left unstimulated or were stimulated with 40 pM TGF-β1 for 16–20 h. All cells were then washed with D-PBS and lysed. Firefly and *Renilla reniformis* luciferase activities were measured using Promega's dual luciferase reporter assay system according to the manufacturer's protocol. Luciferase activity was normalized utilizing the ratio of Firefly to *R. reniformis* luciferase activity and presented as -fold induction. All assays were done in triplicate wells, and each experiment was repeated at least twice.

Wound Closure and Transwell Motility Assays—For wound closure assays, confluent cell monolayers were wounded by manually scraping the cells with a pipette tip. Following wounding, wound size was verified with an ocular ruler to ensure that all wounds were the same width. The cell culture medium was then replaced with fresh medium, and wound closure was monitored by microscopy at various times.

Transwell motility assays were performed utilizing 5-μm pore, 6.5-mm polycarbonate transwell filters (Corning Costar Corp., Cambridge, MA). For these assays, single cell suspensions were seeded in serum-free medium containing 0.1% bovine serum albumin onto the upper surface of the filters and allowed to migrate toward various concentrations of FCS. After a 16–20-h incubation period, cells on the upper surface of the filter were wiped off with a cotton swab, and the cells that had migrated to the underside of the filter were fixed, stained with 0.5% crystal violet, and counted by brightfield microscopy at ×200 in five random fields.

Adenoviral Expression of ALKs and Smads—The adenoviral construct encoding FLAG-tagged Smad4 (39) was obtained from Dr. Harold Moses (Vanderbilt University, Nashville, TN). All other adenoviral constructs encoding FLAG-tagged Smads or HA-tagged constitutively active mutants of the TGF-β (ALK5^{T204D}), activin (ALK2^{Q207D}), and BMP (ALK3^{Q233D}) type I receptors (40) were generously provided by Dr. Kohei Miyazono (Japanese Foundation for Cancer Research, Tokyo, Japan). Stocks of recombinant viruses for each of these constructs were generated in 293 cells and titered utilizing the Takara assay (Takara Biomedicals, Tokyo, Japan). Cells were then infected with these or a control β-galactosidase adenovirus at a multiplicity of infection (m.o.i.) that resulted in >90% cell infection (~15 plaque-forming units/cell or less). The efficiency of infection was evaluated by *in situ* staining of cells for β-galactosidase activity 48 h following infection.

RESULTS

TβRII-K277R Is Expressed in MDA-MB-231 Cells—To abrogate TGF-β signaling in MDA-MB-231 cells, an expression vector encoding GFP and kinase-inactive TβRII was transfected into cells. Expression of the kinase-inactive TβRII-K277R mutant was verified by affinity labeling cell surface receptors with ^{125}I-TGF-β1 (Fig. 1*A*). Because TβRII-K277R has an intact extracellular domain, it can still bind TGF-β and should therefore co-migrate with endogenous TβRII. Cell surface labeling of parental cells resulted in the labeling of three proteins corresponding to the endogenous type I, II, and III TGF-β receptors. There was little or no change in the amount of receptor labeling observed in the control cells expressing GFP alone (GABE 15 Pool) compared with parental cells. However, in the pool expressing TβRII-K277R (dnTβRII 15 Pool),

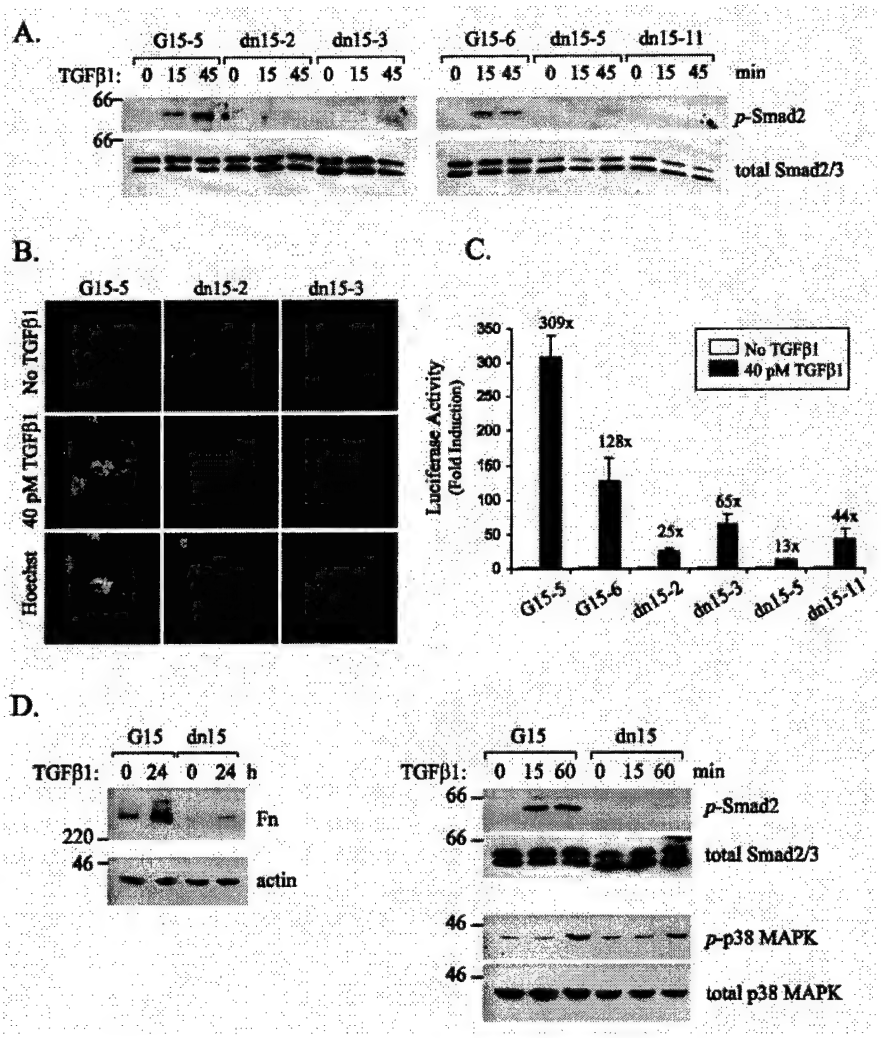

FIG. 3. **Expression of TβRII-K277R impairs TGF-β signaling.** *A*, near confluent clones expressing GFP alone (G15–5 and G15–6) or GFP and TβRII-K277R (dn15–2, dn15–3, and dn15–11) were incubated overnight under serum-free conditions, stimulated with 80 pM TGF-β1 for the times indicated, washed, and lysed. Protein extracts (50 *μg/lane*) were separated by 10% SDS-PAGE followed by immunoblot analysis for phospho-Smad2 (*p-Smad2*) and total Smad2/3. *B*, cells were grown on glass coverslips for 48 h and serum-starved for 16 h followed by treatment with 40 pM TGF-β1 for 1 h. Cells were then prepared for indirect immunofluorescence staining of Smad2. Nuclear localization of Smad2 was confirmed by staining the same cells with Hoechst. *C*, cells were transiently transfected with $p(CAGA)_{12}$-Luciferase along with pCMV-Renilla. The following day, cells were split to six wells of a 24-well plate, treated with 40 pM TGF-β1 for 16 h, washed, and lysed. Firefly and *Renilla* luciferase activities were measured using Promega's dual luciferase reporter assay system. Fold induction of luciferase activity (*y axis*) is based on the ratio of Firefly to *Renilla* luciferase activities. Each *data point* represents the mean ± S.D. of three wells. *D*, near confluent pools expressing GFP alone (*G15*) or GFP and TβRII-K277R (*dn15*) were incubated overnight under serum-free conditions, stimulated with 80 pM TGF-β1 for the times indicated, and prepared for immunoblot analysis as in *A*. The fibronectin blot (*Fn*) was probed with an antibody directed against actin as a loading control, whereas the phospho-Smad2 (*p-Smad2*) and phospho-p38 MAPK (*p-p38 MAPK*) blots were probed with antibodies directed against the unphosphorylated forms of the respective proteins to verify equal loading.

there was a significant increase in the amount of labeled type II receptor, suggesting that the exogenous receptor was expressed. Individual clones obtained from each of these pools expressing either GFP alone (G15–5, -6) or GFP and TβRII-K277R (dn15–2, -3, -5, -11) displayed a similar pattern of labeling.

To confirm that this increase in the labeling of TβRII was indeed because of expression of the HA-tagged TβRII-K277R, extracts from affinity labeled cells were immunoprecipitated with an HA antibody. As shown in Fig. 1*B*, the HA antibody immunoprecipitated a labeled type II receptor in the pool and clones expressing TβRII-K277R but not in the control pool or clones expressing GFP alone, confirming transgene expression. The type I TGF-β receptor appeared to co-immunoprecipitate with TβRII-K277R in these experiments. This was confirmed in subsequent co-immunoprecipitation experiments (see Fig. 2).

TβRII-K277R Is Functional in MDA-MB-231 Cells—Having ascertained that TβRII-K277R was expressed, we then examined whether it was functional. Immunoprecipitation experiments revealed that when affinity labeled cells expressing TβRII-K277R were precipitated with an HA antibody, a labeled protein the size of a type I receptor co-precipitated with TβRII-K277R (see Fig. 1*B* and Fig. 2, *right panel*, *lane 8*). We confirmed that this was TβRI by precipitating similarly labeled cells with various TGF-β superfamily type I receptor antibodies, including ALK1, -2, and -5. Only the ALK5 (TβRI), but not the ALK1 or ALK2 antibodies, precipitated the cross-linked type I receptor (Fig. 2). Although the TβRII antibody co-precipitated ALK5 efficiently, the ALK5 antibody co-precipitated TβRII only weakly (see Fig. 2, *lane 5*, *both panels*). In the control GABE 15 Pool, the HA antibody failed to precipitate any proteins, as expected. Immunoprecipitations with a TβRII antibody were carried out in both pools as a positive control, and both resulted in the co-precipitation of TβRI (Fig. 2, *lane 7*, *both panels*). These data indicate that TβRII-K277R associates with TβRI.

To determine whether TβRII-K277R prevented TGF-β signaling, we examined its effect on the ability of TβRI to phosphorylate Smad2. Immunoblot analysis of TGF-β1-treated cell lysates using a phospho-specific Smad2 antibody revealed that although TGF-β1 could induce phosphorylation of Smad2 in both GABE clones (G15–5 and G15–6), its ability to do so in the TβRII-K277R clones (dn15–2, -3, -5, -11) was impaired (Fig. 3*A*). This impairment was not because of a decrease in total Smad2 protein, as reprobing with an antibody directed against total Smad2/3 did not reveal any significant change in protein levels. We next examined the effect of TβRII-K277R expression on TGF-β-induced Smad translocation to the nucleus by immunofluorescence (Fig. 3*B*). In the GABE clone Smad2 staining was relatively diffuse, but upon TGF-β1 treatment for 60 min, Smad2 staining became concentrated in the nucleus. In contrast, in the TβRII-K277R clones, there was little or no change in Smad2 staining following TGF-β1 treatment, suggesting impaired TGF-β-mediated translocation of Smad2 to the nu-

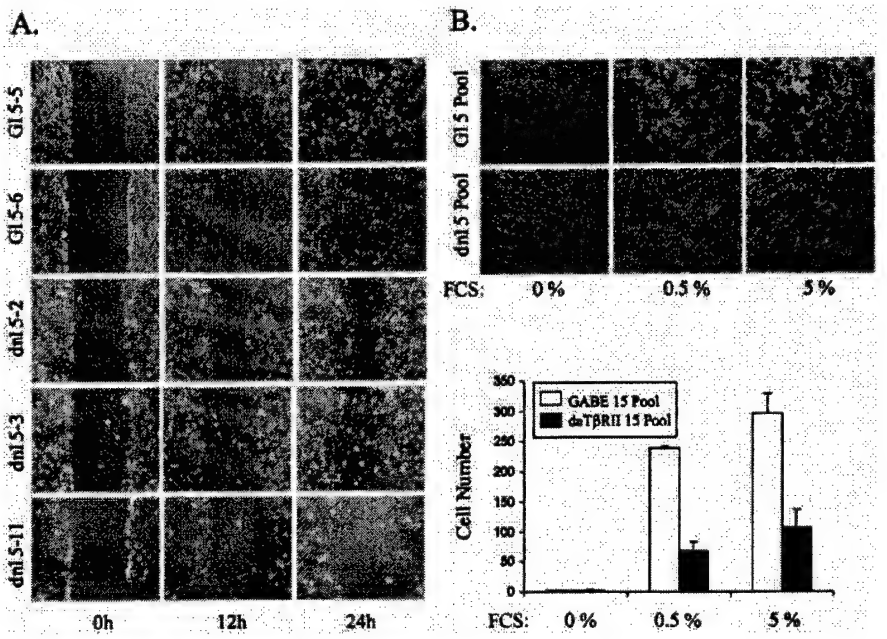

FIG. 4. **Expression of TβRII-K277R impairs motility.** *A*, confluent cell monolayers of clones expressing GFP alone (*G15–5* and *G15–6*) or GFP and TβRII-K277R (*dn15–2*, *dn15–3*, and *dn15–11*) were wounded with a pipette tip. Following wounding, cell culture medium was replaced with fresh medium, and wound closure was monitored by microscopy at the times indicated. *B*, single cell suspensions of pools expressing GFP alone (*G15 Pool*) or GFP and TβRII-K277R (*dn15 Pool*) in serum-free medium containing 0.1% bovine serum albumin were seeded onto 5 μM polycarbonate transwell filters and allowed to migrate toward increasing concentrations of FCS, as indicated. After 20 h, cells on the underside of the filters were fixed, stained, and counted. The results are represented quantitatively in the *bar graph below* the representative *filter micrographs*. Each *data point* represents the mean ± S.D. of two wells.

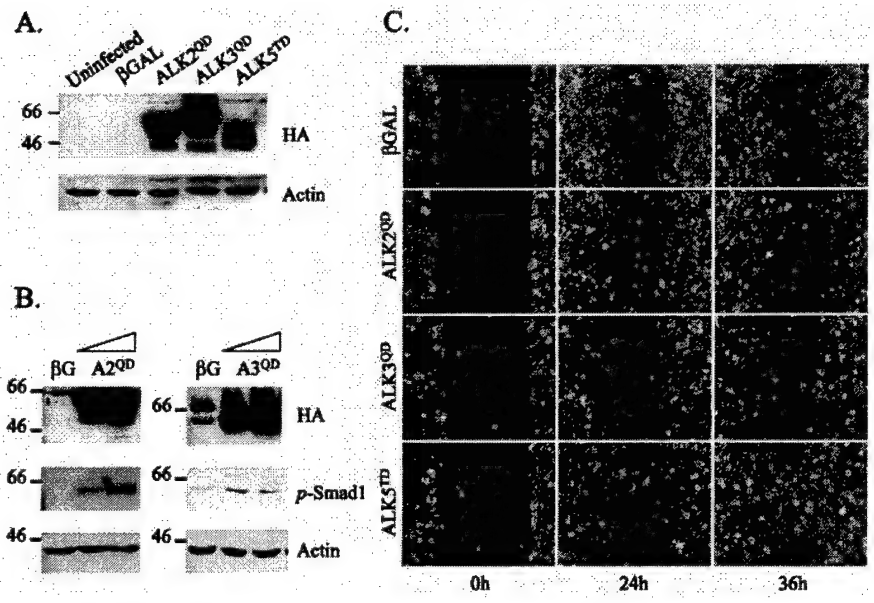

FIG. 5. **Expression of ALK5^{TD}/TβRI restores motility in TβRII-K277R cells.** Clone dn15–2 was infected with adenoviruses encoding HA-tagged constitutively active mutants of the activin (*ALK2^{QD}*), BMP (*ALK3^{QD}*) or TGF-β (*ALK5^{TD}*) type I receptors at an m.o.i. of 15. Uninfected cells and cells infected with a β-galactosidase (*βGAL*) adenovirus at a similar m.o.i. were used as controls. Approximately 48 h following infection, ALK expression (*A*) and function (*B*) were verified by immunoblot analysis utilizing an anti-HA or a phospho-specific Smad1 antibody, as indicated. The blots were also probed with an actin antibody to verify equal loading. The effect of ALK expression on wound closure was monitored by microscopy at the times indicated (*C*).

cleus. We then examined the effect of TβRII-K277R on TGF-β1-induced transcription. A reporter construct containing twelve Smad binding elements repeated in tandem, p(CAGA)$_{12}$-Luciferase, was transiently transfected into the GABE and TβRII-K277R clones, along with pCMV-Renilla. Normalized luciferase activity indicated that TGF-β1 could induce transcription of both reporter constructs in the GABE clones, but its ability to do so in the TβRII-K277R clones was impaired (Fig. 3*C*).

To determine whether signaling pathways other than the Smad pathway were also affected by expression of TβRII-K277R, we examined fibronectin expression, which has been reported to be induced by TGF-β in a JNK-dependent but Smad4-independent manner (6). We chose to perform these experiments in our pools as the results obtained in these cells are representative of those obtained in the clones (compare phospho-Smad2 blots in Fig. 3, *A* and *D*). Following TGF-β stimulation for 24 h, we observed an increase in fibronectin expression in the GABE pool, but this induction was decreased significantly in the pool expressing TβRII-K277R, as was the basal level of fibronectin expression (Fig. 3*D*). We were unable to detect any induction of phosphorylation of JNK in response to TGF-β in our GABE pools (data not shown). However, we did observe an increase in phosphorylation of p38 MAPK following TGF-β stimulation for 60 min, and this induction of phosphorylation was slightly attenuated in the pool expressing TβRII-K277R (Fig. 3*D*).

TβRII-K277R Impairs the Motility of MDA-MB-231 Cells—Next we examined the effect of TβRII-K277R expression on the motility of MDA-MB-231 cells in a wound closure assay. In the GABE clones, cells migrated into the wounded area and closed the wound within 24 h, whereas in the TβRII-K277R clones the wound remained open at 24 h (Fig. 4*A*). This difference in motility did not appear to be because of an effect on proliferation, because when the experiment was performed in the presence of mitomycin C, a compound that inhibits cell division, the same results were obtained (data not shown). Thus, expression

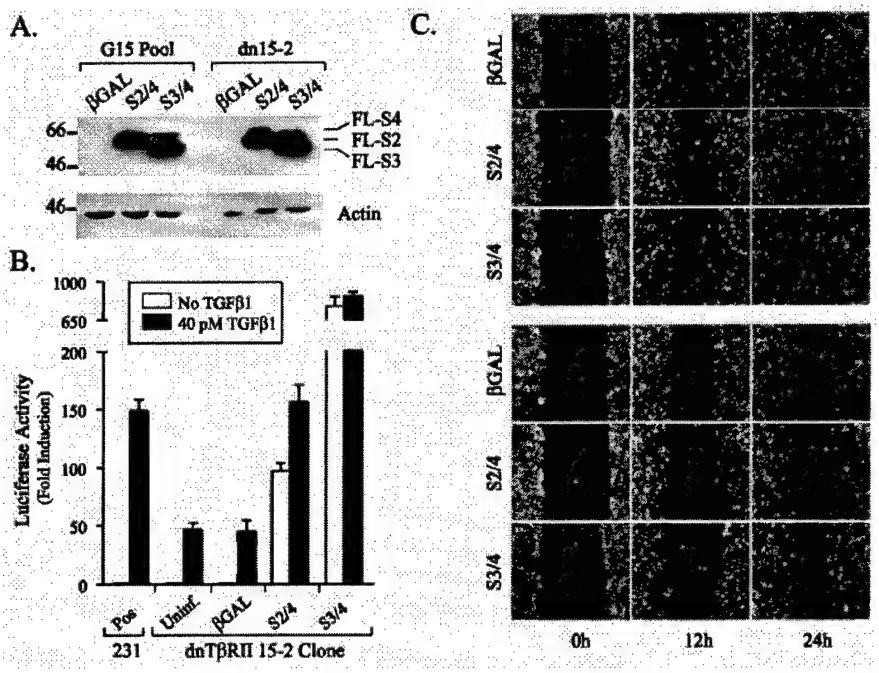

FIG. 6. **Expression of Smad2/4 or Smad3/4 does not restore motility in TβRII-K277R cells.** Cells were infected with both FLAG-tagged Smad2 and Smad4 (*S2/4*) or FLAG-tagged Smad3 and Smad4 (*S3/4*) encoding adenoviruses at an m.o.i. of 3 for Smad2/3 and an m.o.i. of 15 for Smad4. Uninfected cells (*Uninf.*) and cells infected with a β-galactosidase (*βGAL*) adenovirus were used as controls. Approximately 48 h following infection, Smad expression was verified by immunoblot analysis utilizing an anti-FLAG antibody (*A*). The ability of Smads to restore TGF-β signaling in dn15–2 was evaluated in transcription reporter assays utilizing the TGF-β responsive transcription reporter $p(CAGA)_{12}$-Luciferase (*B*). The effect of Smad expression on wound closure in the control G15 Pool (*top panel*) and dn15–2 clone (*bottom panel*) was monitored by microscopy at the times indicated (*C*).

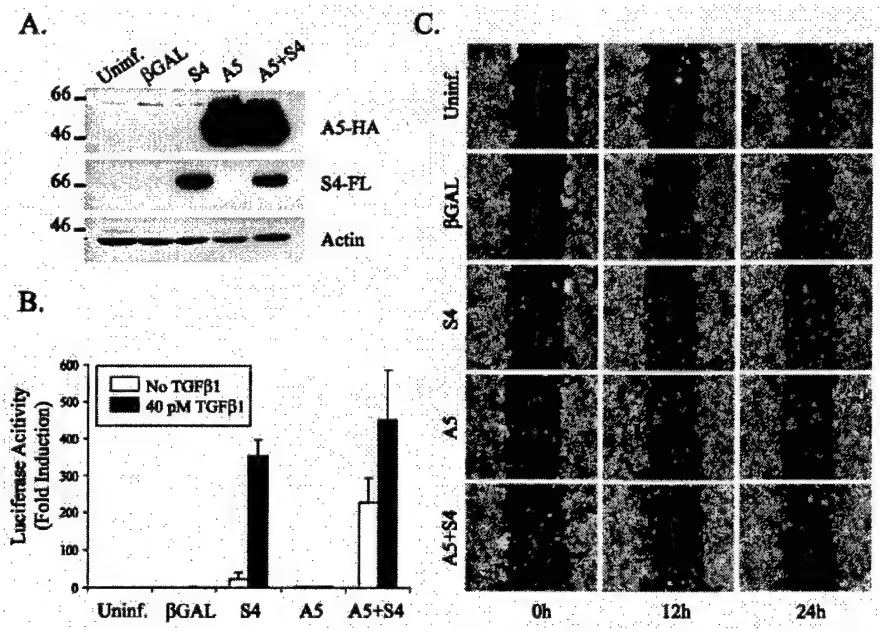

FIG. 7. **Effect of $ALK5^{TD}$ on the motility of MDA-MB-468 cells −/+ Smad4.** Cells were infected with adenoviruses encoding FLAG-tagged Smad4 (*S4*), HA-tagged $ALK5^{TD}$ (*A5*), or both (*A5+S4*) at an m.o.i. of 15. Uninfected cells (*Uninf.*) and cells infected with a β-galactosidase (*βGAL*) adenovirus were used as controls. Approximately 48 h following infection, exogenous Smad4 and $ALK5^{TD}$ expression was verified by immunoblot analysis utilizing anti-FLAG and anti-HA antibodies, respectively. The blots were also probed with an actin antibody to verify equal loading (*A*). The ability of Smad4 and $ALK5^{TD}$ to activate Smad-dependent signaling was evaluated in transcriptional reporter assays utilizing the Smad-dependent $p(CAGA)_{12}$-Luciferase reporter construct (*B*), and their effect on motility was examined in wound closure assays (*C*).

of TβRII-K277R in MDA-MB-231 cells appears to impair their motility, independent of changes in proliferation. As an alternative measure of cell motility, we also examined the effect of TβRII-K277R on the ability of cells to migrate in a transwell assay system. We observed a 3- to 4-fold reduction in the ability of cells expressing TβRII-K277R to migrate toward FCS, as compared with control cells expressing GFP alone (Fig. 4*B*).

The Impaired Motility of TβRII-K277R Cells Is TGF-β Type I Receptor-specific—Because the impaired motility of TβRII-K277R cells was observed in the absence of exogenous TGF-β stimulation, we wished to determine whether this impairment was TGF-β-specific. To do so, we chose to restore TGF-β signaling in TβRII-K277R cells by expressing a constitutively active mutant of TβRI. Mutation of threonine 204 in ALK5 (TβRI) to aspartic acid leads to constitutive activation of the type I receptor kinase, allowing it to induce signals in the absence of ligands or type II receptors (41). Likewise, mutation of corresponding threonine and glutamine residues in the activin (42) and BMP (43) type I receptors to aspartic acid also leads to constitutive activation of these kinases. To test for TGF-β specificity, cells expressing TβRII-K277R were infected with adenoviruses encoding HA-tagged constitutively active mutants of the TGF-β ($ALK5^{TD}$), activin ($ALK2^{QD}$), and BMP ($ALK3^{QD}$) type I receptors (40). Uninfected cells or cells infected with a β-galactosidase adenovirus at the same m.o.i. were used as controls. The efficiency of infection was >90% as evaluated by *in situ* staining of cells for β-galactosidase activity 48 h following infection (data not shown). At this time, expression of the mutant type I receptors was confirmed by immunoblot analysis utilizing an HA antibody (Fig. 5*A*), and their effect on motility was assessed in wound closure assays. Motility was only restored in cells expressing $ALK5^{TD}$ (Fig. 5*C*). Although

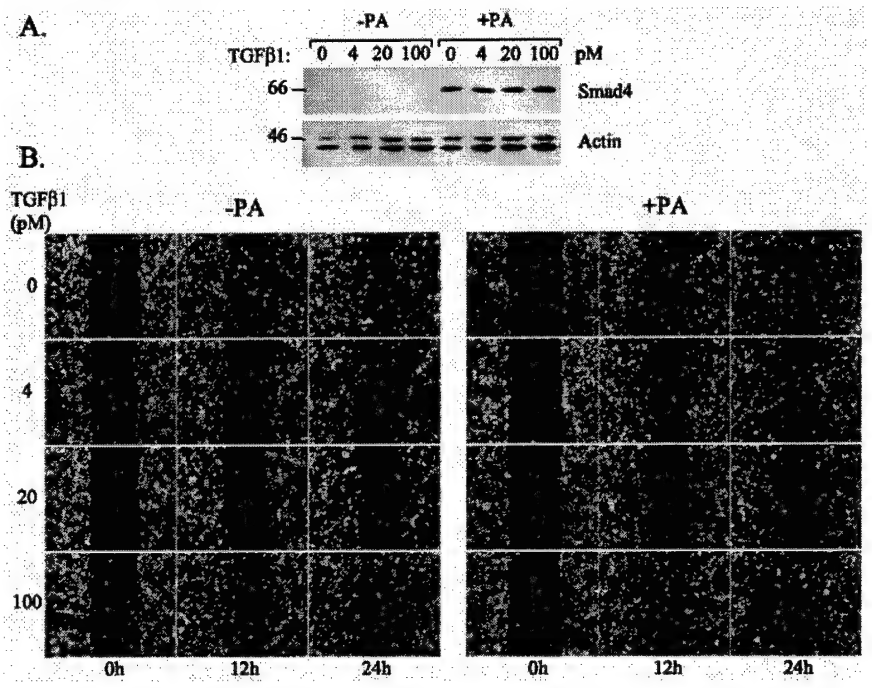

FIG. 8. **Effect of exogenous TGF-β1 stimulation on the motility of SW480.7 cells −/+ Smad4.** Cells in 6-well plates were either left untreated (*left panel*) or treated with 3 μM ponasterone (*right panel*) for 40 h to induce Smad4 expression. Cells were then wounded, washed, and incubated with serum-free medium in the presence of 0, 4, 20, or 100 pM TGF-β1 for 24 h. Wound closure was monitored by microscopy at the times indicated (*B*). At the conclusion of the wound closure experiment, cells were lysed, and Smad4 expression was examined by immunoblot analysis utilizing a monoclonal antibody directed against Smad4 (*A*). The blots were also probed with an actin antibody to verify equal loading. Ponasterone was maintained in the culture medium of selected wells throughout the experiment.

$ALK2^{QD}$ and $ALK3^{QD}$ were expressed and functional, as evidenced by their ability to induce Smad1 phosphorylation (Fig. 5*B*), they failed to restore motility in cells expressing TβRII-K277R (Fig. 5*C*). These results suggest that the impaired motility of TβRII-K277R cells is TβRI (ALK5)-specific.

Restoration of Smad Signaling Does Not Rescue the Impaired Motility of TβRII-K277R Cells—Although there is evidence that Smad signaling is critical for the anti-proliferative effects mediated by TGF-β (44, 45), it is unclear whether TGF-β-mediated motility requires Smad signaling. We reasoned that if Smads are required for TGF-β-mediated motility, blockade of Smad signaling with dominant-negative Smad mutants or with the inhibitory Smad, Smad7, should impair motility. However, expression of either dominant-negative Smad4 or Smad7 in the MDA-MB-231 parental cells resulted in cell death (data not shown). Therefore, it was not possible to address whether TGF-β-mediated motility requires Smad signaling utilizing this approach. Instead, we chose to overexpress the TGF-β R-Smads, Smad2 or Smad3, along with Smad4, in cells expressing TβRII-K277R to determine whether reconstitution of Smad signaling could restore motility. Cells were infected with adenoviruses encoding FLAG-tagged Smad2 and Smad4 or FLAG-tagged Smad3 and Smad4, and exogenous Smad expression was confirmed by immunoblot analysis utilizing an anti-FLAG antibody (Fig. 6*A*). The ability of Smad2/4 and Smad3/4 to activate Smad-dependent signaling was examined utilizing the Smad-dependent transcription reporter construct, $p(CAGA)_{12}$-Luciferase. Expression of Smad3/4 resulted in a marked increase in basal transcription (Fig. 6*B*). Stimulation with TGF-β1 did not cause any further increase in transcription, suggesting that Smad signaling was activated maximally. Despite this, Smad3/4 failed to restore motility in the cells expressing TβRII-K277R (Fig. 6*C*, *bottom panel*) and had no effect on the motility of control cells expressing GFP alone (Fig. 6*C*, *top panel*). Activation of basal transcription was not as marked with Smad2/4, as expected, because Smad2 itself cannot bind DNA (46). However, despite nearly 100-fold induction of transcription following infection at the maximally tolerated m.o.i., this combination also failed to restore motility in the cells expressing TβRII-K277R (Fig. 6*C*, *bottom panel*) and had no effect on the motility of control cells expressing GFP alone (Fig. 6*C*, *top panel*). These results indicate that reconstitution of Smad signaling alone is not sufficient to restore autocrine TGF-β-mediated motility in cells expressing TβRII-K277R, nor is it sufficient to enhance the motility of control MDA-MB-231 cells.

Re-expression of Smad4 in Smad4-defective Cancer Cells Does Not Enhance Motility—To determine whether Smads are required for cancer cell migration, we examined whether activation of TGF-β signaling could promote motility in the absence of Smad signaling utilizing Smad4 null MDA-MB-468 breast cancer cells (47). Smad4 and $ALK5^{TD}$ were expressed, either alone or in combination, by adenoviral transduction, and their effects on the motility of MDA-MB-468 cells were examined in wound closure assays. Expression of HA-tagged $ALK5^{TD}$ and FLAG-tagged Smad4 was confirmed by immunoblot analysis (Fig. 7*A*), and their ability to activate Smad-dependent signaling was examined in transcription reporter assays utilizing the Smad-dependent $p(CAGA)_{12}$-Luciferase reporter construct (Fig. 7*B*). As expected, in the absence of Smad4 (uninfected, β-galactosidase, $ALK5^{TD}$ alone), TGF-β1 was unable to stimulate transcription in these cells. However, upon re-expression of Smad4 a marked increase in both TGF-β1-mediated and $ALK5^{TD}$-mediated transcription was observed, indicating that both Smad4 and $ALK5^{TD}$ were indeed functional in these cells. Despite this, neither Smad4 nor $ALK5^{TD}$ had any effect on cell motility, whether they were expressed alone or in combination (Fig. 7*C*). The fact that $ALK5^{TD}$ could not promote motility, even when Smad4 was co-expressed with it, suggests that MDA-MB-468 cells are not responsive to the pro-migratory effects of TGF-β.

To determine whether TGF-β could induce migration in the absence of Smad signaling in other cells, the Smad4 defective SW480.7 colorectal cells, conditionally expressing Smad4 via an ecdysone-inducible system (34), were utilized. Cells were stimulated with increasing concentrations of TGF-β1 in the absence or presence of 3 μM ponasterone to induce Smad4 expression. Smad4 expression in ponasterone-treated cells was confirmed by immunoblot analysis (Fig. 8*A*), and its effect on TGF-β-mediated motility was examined in wound closure assays. Again, as in the MDA-MB-468 cells, these cells failed to

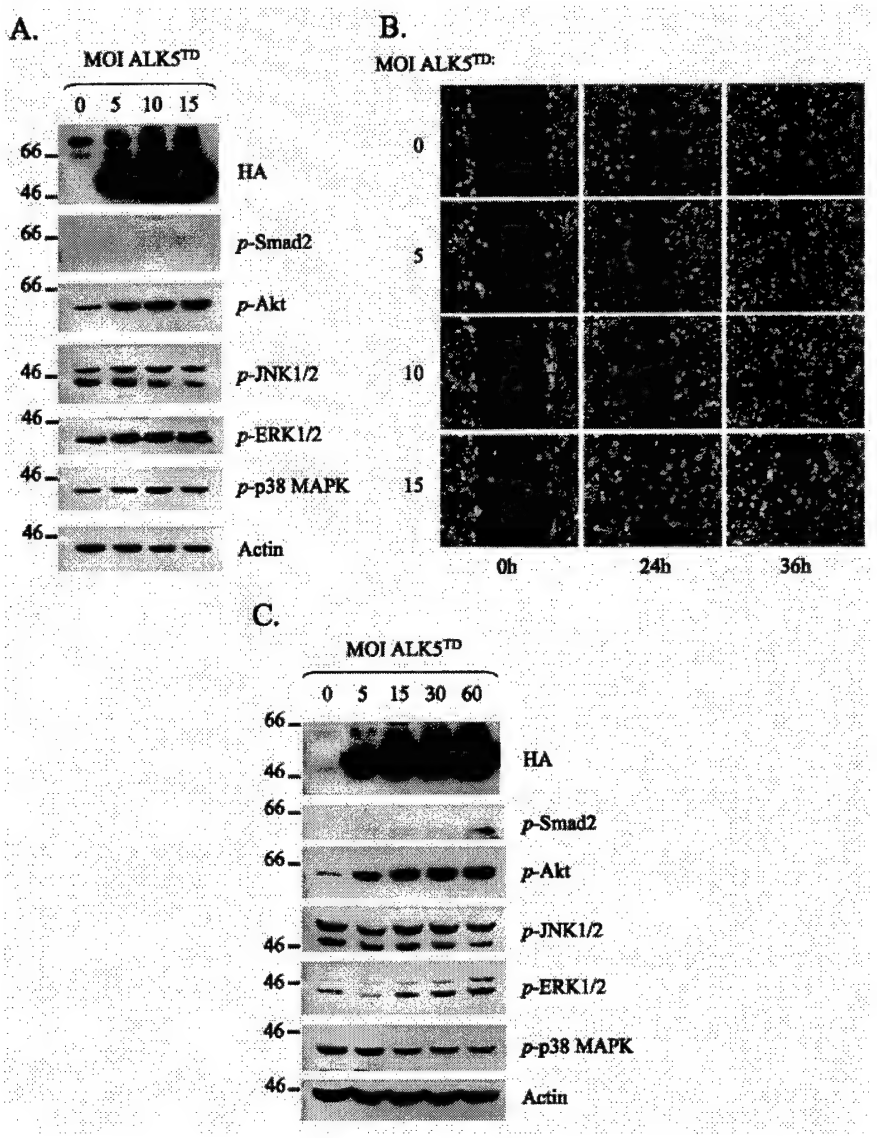

FIG. 9. **Different signaling pathways require different thresholds of TGF-β activation.** Clone dn15–2 was infected with an adenovirus encoding an HA-tagged constitutively active mutant of the TGF-β type I receptor (*ALK5^{TD}*) at m.o.i. values of 0, 5, 10, or 15, as indicated. Approximately 48 h following infection, cells were wounded, and the effect of ALK5^{TD} expression on wound closure was monitored by microscopy at the times indicated (*B*). At the conclusion of the migration assay, cells were lysed, ALK5^{TD} expression was confirmed by HA immunoblot, and the activation status of candidate signaling pathways was examined utilizing phospho-specific antibodies, as indicated (*A*). Actin was examined as a loading control. In *C*, clone dn15–2 was re-infected with ALK5^{TD} at m.o.i. values of 0, 5, 15, 30, or 60, as indicated, and the activation status of candidate signaling pathways was re-examined utilizing phospho-specific antibodies as in *A*.

respond to TGF-β both in the absence and presence of Smad4 (Fig. 8*B*). Taken together, these data indicate that reconstitution of Smad signaling alone is not sufficient to promote migration of cancer cells.

Different Signaling Pathways Require Different Thresholds of TGF-β Activation—To determine whether Smad signaling is actually required in addition to other pathways activated by TGF-β to promote migration, we examined what signaling pathways were activated under conditions where motility was restored following ALK5^{TD} expression in cells expressing TβRII-K277R (see Fig. 5*C* and Fig. 9*B*). For these experiments, expression of ALK5^{TD} was confirmed by HA immunoblot (Fig. 9*A*), and the activation status of candidate signaling pathways was examined utilizing phospho-specific antibodies. Under these conditions, we observed an increase in the phosphorylation of Akt and ERK1/2, with little or no change in the phosphorylation of JNK or p38 MAPK (Fig. 9*A*). Interestingly, the levels of ALK5^{TD} expression that were sufficient to restore motility were not sufficient to induce phosphorylation of Smad2. Additional experiments indicated that ~4-fold greater ALK5^{TD} expression was required for induction of Smad2 phosphorylation (Fig. 9*C*). At that m.o.i., the viral load *per se* interfered with the ability of the cells to migrate. These data indicate that different signaling pathways require different thresholds of TGF-β activation, as do different biological effects mediated by TGF-β (27), and suggest that TGF-β may promote motility through mechanisms independent of Smad signaling, possibly involving activation of the PI3K-Akt and/or MAPK pathways. Consistent with this, the ability of TGF-β to promote migration was impaired in the presence of the PI3K inhibitor, LY294002 (Fig. 10*A*), as well as in the presence of JNK, MEK, and p38 MAPK pathway inhibitors (Fig. 10*B*).

DISCUSSION

In this study, abrogation of autocrine TGF-β signaling in MDA-MB-231 breast cancer cells resulted in an impairment in basal cell migration, which could not be restored by reconstituting Smad signaling, suggesting that Smad signaling alone is not sufficient for autocrine TGF-β-mediated motility. Consistent with this, reconstitution of Smad signaling in the Smad4-defective MDA-MB-468 and SW480.7 cells did not promote migration. In addition, restoration of migration following restoration of TGF-β signaling in cells expressing TβRII-K277R was associated with an increase in phosphorylation of Akt and ERK1/2 but not Smad2. These results indicate that Smad signaling is dispensable for TGF-β-mediated motility and that

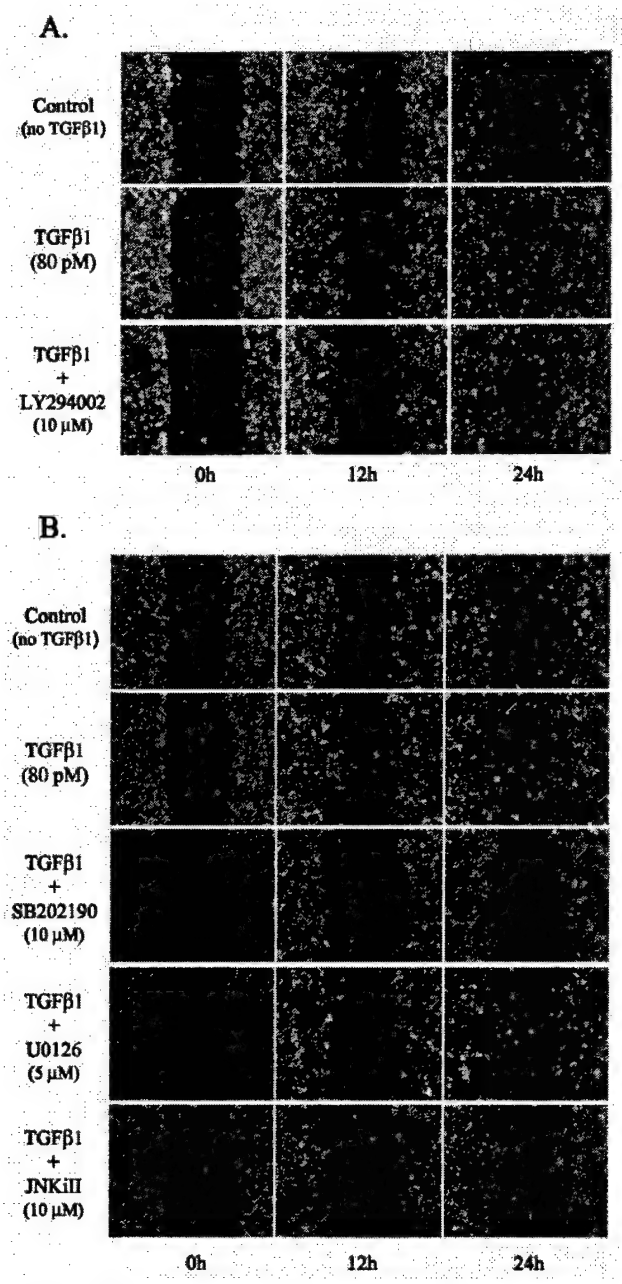

FIG. 10. **TGF-β-induced motility requires activation of multiple signaling pathways.** Confluent MDA-MB-231 cell monolayers were wounded with a pipette tip. Following wounding, cells were washed, the cell culture medium was replaced with serum-free medium, and the cells were stimulated with 80 pM TGF-β1 in the absence or presence of the PI3K inhibitor, LY294002 (*A*) or the p38 MAPK (*SB202190*), MEK (*UO126*), and JNK (*JNKiII*) inhibitors, as indicated (*B*). Wound closure was monitored by microscopy at the times indicated.

this response is instead mediated through alternative pathways activated by TGF-β. In support of this, the ability of TGF-β to promote migration was blocked in the presence of pharmacological inhibitors of the PI3K, p38 MAPK, MEK, and JNK pathways.

Although Smads have been implicated as critical mediators of many TGF-β responses (48–52), the role of Smads in cancer cell migration has, to the best of our knowledge, not been reported. Previous studies in non-transformed cells have generated conflicting data on the requirement of Smad signaling for cell migration (50, 53–55). In one study, expression of dominant-negative Smad3 in non-transformed murine mammary cells had no effect on TGF-β-mediated motility even though it blocked the anti-mitogenic effect of TGF-β (53). This suggests that Smad3 is not required for this response or that residual Smad3 signaling, not blocked by expression of dominant-negative Smad3, is sufficient to mediate motility. This would be consistent with the idea that different biological responses require different thresholds of TGF-β signaling (27). Thus, complete abrogation of Smad3 signaling might be required to observe an impairment in TGF-β-mediated motility whereas partial blockade of Smad function might be sufficient to block the anti-proliferative effects of TGF-β.

Interestingly, expression of either dnSmad4 or antagonistic Smad7 in MDA-MB-231 cells resulted in cell death. Although overexpression of Smad7 has been reported to sensitize various cell types to cell death (56), expression of dnSmad4 has not been associated with such a response. There is, however, increasing evidence that TGF-β can promote the survival of both transformed (7, 57, 58) and non-transformed (59–61) cells. Whether Smad signaling is required for these pro-survival effects of TGF-β is not known. In addition, re-expression of Smad4 in Smad4-defective SW480 has been reported to induce a more adhesive and flat phenotype (62). These results suggest that blockade of Smad signaling could potentially lead to loss of adhesion and result in anoikis. This could explain our inability to express dnSmad4 and Smad7 in MDA-MB-231 cells.

Because we were unable to assess the requirement for Smads in autocrine TGF-β-mediated motility by abrogating Smad signaling, we chose to address this question by activating Smad signaling in cells expressing TβRII-K277R. Having ascertained that the impaired motility of TβRII-K277R cells was indeed TβRI-specific, we overexpressed the TGF-β R-Smads, Smad2 or Smad3, each with Smad4, to determine whether autocrine TGF-β-mediated motility was Smad-dependent. Despite their ability to activate Smad-dependent transcription, neither Smad combination restored the impaired motility of the TβRII-K277R cells. We (data not shown) and others (62) have observed an increase in cell spreading following Smad overexpression. It is tempting to speculate that this increased cell spreading may be associated with increased adhesion, which interferes with cell migration. This could potentially explain why restoration of Smad signaling in TβRII-K277R cells failed to restore migration. These data suggest that in breast cancer cells, autocrine TGF-β signaling mediates motility in a Smad-independent manner or that alternative pathways, in addition to the Smad signaling pathway, are required for these effects.

To address this question, we examined what signaling pathways were activated under conditions where motility was restored in TβRII-K277R cells following expression of a constitutively active type I TGF-β receptor. In these experiments, we observed an increase in the phosphorylation of Akt and ERK1/2 but not Smad2. These data further imply that Smad signaling is not required for TGF-β-mediated motility. Although expression of $ALK5^{TD}$ may induce a low level of Smad phosphorylation, which cannot be detected by immunoblot analysis, it is unlikely that Smads are required for autocrine TGF-β-mediated motility as reconstitution of Smad signaling in both MDA-MB-468 and SW480.7 cancer cell lines failed to promote motility, and expression of both low and high levels of either Smad2/4 or Smad3/4 failed to rescue the impaired motility of MDA-MB-231 cells expressing dnTβRII. Thus, alternative signaling pathways activated by TGF-β are more likely to be important for migration. Indeed, blockade of the PI3K, p38 MAPK, MEK, and JNK pathways with pharmacological inhibitors impaired TGF-β-stimulated migration. The fact that inhibitors of p38 MAPK and JNK interfered with TGF-β-induced

migration even though $ALK5^{TD}$ failed to alter their phosphorylation status suggests that these signaling pathways, though not activated further by TGF-β in our experimental system, are required for basal cell migration. In agreement with this, we have indeed observed an impairment in the basal migratory potential of these cells in the presence of these inhibitors (data not shown).

The observation that different levels of $ALK5^{TD}$ expression resulted in differential activation of downstream targets (Fig. 9*C*) indicates that different signaling pathways require different thresholds of TGF-β activation. In agreement with this, others have reported that expression of dnTβRII in NMuMG mammary cells impairs TGF-β-mediated Smad-dependent inhibition of proliferation but not TGF-β-mediated activation of p38 MAPK (9). In addition, there is evidence that different biological responses mediated by TGF-β also require different thresholds of TGF-β signaling. For example, expression of dnTβRII in squamous carcinoma cells has been reported to block the growth inhibitory effects of TGF-β but not its ability to induce EMT (26). Likewise, expression of dnTβRII in 4T1 murine mammary cancer cells impairs TGF-β-mediated transcription but fails to block motility (27). Because TGF-β signaling was not completely abrogated in the squamous and mammary cancer cells (26, 27), the molecular mechanisms by which autocrine TGF-β may selectively contribute to tumor progression could not be fully addressed in those studies. Because we have expressed TβRII-K277R at levels high enough to block both Smad and non-Smad pathways in MDA-MB-231 cells, the model we have generated should prove useful in dissecting the signaling pathways required for the diverse effects elicited by TGF-β in cancer.

Our data indicate that autocrine TGF-β-mediated motility of cancer cells is Smad-independent. This implies that non-transformed cells and transformed cells utilize different mechanisms to promote motility as others have reported that Smad3 null monocytes and keratinocytes exhibit significantly reduced migration to TGF-β1 in transwell motility assays (54). Moreover, Smad3 appears to be required for TGF-β-mediated monocyte chemotaxis *in vivo*, as mice lacking the Smad3 gene display a blunted monocyte chemotactic response following cutaneous wounding (54). Studies in *Drosophila* also suggest that Smads may be required for cell migration as mutations in Mad, the *Drosophila* receptor-activated Smad, impair migration of the epidermis during dorsal closure (55). Finally, recent studies in endothelial cells have indicated that TGF-β acting through ALK1 stimulates migration in a Smad-dependent manner, whereas TGF-β acting through ALK5 inhibits cell migration in a Smad-dependent manner (50). Taken together, these studies highlight the importance of Smads in TGF-β-regulated migration of non-transformed cells.

Despite compelling evidence for the role of Smads in non-transformed cell migration, a lack of requirement for Smad signaling in TGF-β-mediated cancer cell migration is consistent with previous studies that have shown that TGF-β can increase cellular motility of prostate cancer cells without affecting proliferation, suggesting that the effects on motility and proliferation may occur via different biochemical pathways (28). Likewise, expression of Smad7 in pancreatic cancer cells has been shown to abrogate the anti-proliferative effects of TGF-β but enhance matrix-associated transcriptional responses, highlighting a dissociation between the matrix and anti-proliferative effects induced by TGF-β (63). If the biological effects of TGF-β that can contribute to tumor progression were Smad-independent, it might be possible to selectively disrupt those pathways, while ensuring that the tumor suppressive, Smad-dependent pathways are maintained. The signaling pathways currently implicated in mediating the various pro- and anti-tumorigenic effects of TGF-β indicate that this may in fact be possible. For example, recent studies aimed at identifying the mechanisms by which TGF-β1 elicits EMT in mammary cells have indicated that the PI3K, RhoA, and p38 MAPK pathways are involved in this process (8, 9, 11, 53). However, whether Smad signaling, which has been implicated in both the anti-proliferative (44, 45) and pro-apoptotic (64, 65) effects of TGF-β, is also required for TGF-β-mediated EMT is unclear. In one study, adenoviral expression of low levels of constitutively active ALK5 induced EMT only if Smad2/4 or Smad3/4 were co-expressed (66). In contrast, other investigators have reported that inhibition of Smad signaling either by overexpression of Smad7 or dominant-negative Smad3 did not affect the transdifferentiation, arguing against the involvement of Smads in EMT (53). Because epithelial transdifferentiation to a mesenchymal phenotype is often associated with acquisition of motile properties, the mechanisms through which TGF-β mediates EMT may be similar to those required for TGF-β-mediated motility. Indeed, the PI3K, RhoA, and p38 MAPK signaling pathways, which are required for TGF-β-mediated EMT, have also been implicated in TGF-β-mediated motility (8, 11, 53). Likewise, we have observed that blockade of these and other pathways interfere with TGF-β-induced motility (Fig. 10), suggesting that multiple pathways cooperate to elicit this effect. It will be of interest to determine whether Smad signaling is required for other effects mediated by TGF-β, as a dissociation between the pathways required for the tumor suppressive *versus* the tumor promoting effects of TGF-β could lead to opportunities to selectively inhibit the non-desirable effects of TGF-β without compromising its tumor suppressive function.

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The TGFbeta superfamily in the pathogenesis of cancer and other diseases, Special AACR Meeting, January 15-19, 2003 La Jolla, CA

Abstract

p38Mapk, Sp1 and Smads contribute to TGFβ-induced expression of fibronectin

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Transforming growth factor beta (TGF-b) is critically involved in tumor invasion, metastasis, cell survival, wound healing, development and the immune responses. TGF-b regulates these events by affecting cell-cell and cell-extracellular matrix contacts, changes in cell morphology, and cell migration. Using cDNA microarray analysis, we identified several components of cell-extracellular matrix contacts whose expression was regulated by TGF-b in normal and tumor cells. In metastatic cells, TGF-b stimulated expression of fibronectin (FN) and integrin beta1 as well as increased Fak1 and Pax phosporylation, indicative of integrin signaling. We investigated the mechanism of TGFβ-induced FN expression in normal and tumor cells. The data suggest that p38Mapk kinase is required for FN expression. Dominant-negative TβRII and Alk5/TβRI blocked TGFβ-mediated activation of p38Mapk and expression of fibronectin. p38Mapk pathway inhibitors markedly reduced FN expression at the mRNA and protein levels. Inhibition of p38Mapk pathway by kinase-inactive MKK3AL, p38AGF or pharmacological inhibitors blocked TGFβ-induced activity of a FN reporter in transient transfection experiments. Analysis of various transcription factors showed that only Smad3 and Smad4 transcription factors significantly stimulated FN promoter activity. The blockade of Sp1 transcription factor binding to DNA by mithramycin A inhibited FN production and blocked integrin signaling, suggesting a critical role for Sp1 in FN expression. Cells with reduced Smad3 or Smad4 expression showed a reduced level of FN promoter activity and FN expression. Together, the presented data suggest that p38Mapk, Sp1, and Smads contribute to TGFβ-induced fibronectin expression leading to activation of integrin signaling.

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Annual AACR Meeting, July 2003, Washington DC

Abstract #102164

TGFβ regulates expression of target genes and p38Mapk signaling to induce changes in the actin cytoskeleton.

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Transforming growth factor beta (TGFβ) is critically involved in tumor invasion, metastasis, cell survival, wound healing, development and the immune responses. TGFβ regulates these events by affecting cell-cell and cell-extracellular matrix contacts, changes in cell morphology, and cell migration. The actin cytoskeleton plays an essential role in these TGFβ responses. We show that TGFβ induces the dynamical changes in the actin cytoskeleton in normal and tumor cells. An initial response involves the membrane ruffling and activation of Rac1 signaling followed by actin stress fiber (SF) formation. The mechanism of these responses is largely unknown. We present data demonstrating that SF formation depends on p38Mapk signaling and the de-novo protein synthesis. Inhibition of p38Mapk affected both membrane ruffling and stress fiber formation in normal and tumor cells. Using cDNA microarray analysis we identified several TGFβ-induced genes (TIGs) involved in the actin polymerization. The functional studies suggest a critical role for several TIGs in the formation of actin SFs. Cells expressing constitutively these TIGs showed extensive SFs independent of the TGFβ presence. Normal and tumor cells that expressed the TIGs in response to TGFβ formed actin SFs, whereas metastatic cancer cells that did not express the TIGs did not formed SFs. The migration of these metastatic cancer cells was strongly stimulated by TGFβ and could be blocked by inhibitors of p38Mapk and ERK1/2 signaling. The ability of TGFβ to induce SFs in metastatic cancer cells was restored by inhibition of ERK1/2 signaling indicating that ERK1/2 signaling negatively regulates SFs formation in metastatic cells. In normal epithelial and non-metastatic tumor cells, TGFβ strongly activates p38Mapk and stimulates expression of TIGs leading to actin SF formation. In metastatic cells, the ability of TGFβ to induce SFs is markedly diminished due to deregulation of TIGs and reduction of p38Mapk signaling. These data suggests that the balance in activities of ERK1/2 and p38Mapk signaling may govern the cell migration. The fact that p38Mapk inhibitors blocked cell migration indicates that a certain level of p38Mapk signaling is required for cell migration. This study suggests a potential anti-metastatic strategy may include inhibition of Ras-ERK1/2 signaling and enhancement of TGFβ signaling to increase expression of TIGs involved in actin SF formation.

This work was supported in part by PHS grant R01 CA62212, DOD grant BC011342, ACS grant #IRG-58-009-43 (to A.V.B.), and Vanderbilt-Ingram Cancer Center NCI support grant CA68485.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Photocopy this page or follow this format for each person.

NAME	POSITION TITLE
Andrei V. Bakin	Assistant Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(If applicable)*	YEAR(s)	FIELD OF STUDY
Moscow State University, Moscow, Russia	M.S.	1980-84	Chemistry/Biochem
Moscow State University, Moscow, Russia	Ph.D.	1985-90	Molecular Biology

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

Research and Professional Experience

1990-1991 Research Fellow, A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow USSR

1990-1991 Visiting Scientist, Department of Chemistry, University of Texas, Austin, TX

1991-1995 Postdoctoral Fellow, Roche Institute of Molecular Biology, Nutley, NJ

1995-1999 Postdoctoral Fellow, Department of Developmental Neurobiology, St. Jude Children's Research Hospital, Memphis, TN

1999-2003 Research Assistant Professor, Dept. of Medicine, Vanderbilt University, Nashville, TN

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Selected Publications (from a total of 27, excluding abstracts)

1. Bakin, A.V., Borisova, O.F., Shatsky, I.N., and Bogdanov, A.A., (1991) Spatial organization of template polynucleotides on the ribosome determined by fluorescent methods. J. Mol. Biol. 221, pp. 441-453.

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